IMMUNE REACTION FOLLOWING INTOXICATION WITH *CLOSTRIDIUM PERFRINGENS* TYPE A ENTEROTOXIN

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This dissertation is dedicated to my supportive wife, Joy, my wonderful new son, Christopher, and to my parents, especially my mother who read to me when I was a child.

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

IMMUNE REACTIONS FOLLOWING INTOXICATION WITH *CLOSTRIDIUM PERFRINGENS* TYPE ENTEROTOXIN

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Many bacterial toxins have been found to interact with the immune system in a variety of ways. Some of these effects prove to be damaging to those exposed to these products. The bacterial pathogen *Clostridium perfringens* is the third leading cause of foodborne illness in the United States. The agent directly responsible for this illness is the *Clostridium perfringens* type A enterotoxin (CPE) which causes a profuse diarrheal illness frequently with symptoms of nausea that are usually self-limiting. Another illness which can be caused by this organism is the infectious diarrhea syndrome most often seen in institutionalized patients such as those in nursing homes. This illness is more protracted than the normal food poisoning and as it frequently strikes the debilitated (ill and/or elderly), it can cause death. It has been generally accepted that the ability of this toxin to cause pore formation in susceptible cells is the mechanism by which diarrhea and other effects of this toxin are caused; however, some bacterial toxins produce similar symptoms by interacting with the immune system. This research determined the effect of CPE upon the immune system using mice as a model system. Following administration of CPE there was a demonstrable interaction with the immune system which could be responsible for some of the symptoms in those affected by this toxin.

CHAPTER 1
INTRODUCTION

Clostridium perfringens type A enterotoxin (CPE) is the causative agent of the third most common cause of food-borne illness (FBI) in the United States, causing greater than 12% of reported cases of FBI. Symptoms of this FBI include a profuse diarrhea which, in severe cases, can be explosive, severe abdominal pain, and nausea. This illness is responsible for much suffering and an estimated economic cost of greater than $200 million in the USA and Canada. Studies have also implicated CPE in many cases of the Sudden Infant Death Syndrome (SIDS). Of the approximately 5,000 cases of SIDS in the USA per year, 50-80% are associated with high levels of CPE and *C. perfringens* spores in the bowel (Lindsay, 1996). Though a cause and effect relationship has not been conclusively proven, the finding of evidence of an immune activation in many cases of SIDS makes this a compelling hypothesis (Vege et al., 1995). Recent research (Bowness et al., 1992) indicated that CPE was a superantigen. Though this subsequently proved incorrect (Krakauer et al., 1997; Wallace et al., 1999), it did lead to additional investigation into the interaction of the immune system and CPE. Further research demonstrated a marked activation of the immune system in a murine model of CPE enteric illness, and suggests several additional avenues of research that may provide further insights into the nature of the FBI associated with CPE, as well as the ultimate causes of SIDS.

The responses of the immune system following exposure to bacterial products have been the subject of much recent research. The reason for this interest is that researchers are now coming to recognize a connection between the immune response and pathology associated with bacterial toxins. In light of this realization, the immune response to CPE was characterized in the mouse model and areas for further research were delineated. Also an investigation into some relevant aspects of SIDS was conducted evaluating whether altered fecal bile acid levels played a role in SIDS and whether neopterin, a product of activated macrophages, was present in increased amounts in SIDS victims. The specific objectives of this study were as follows:

1. To determine whether CPE was, as reported, a superantigen.
2. To determine the in vitro cytokine response of a macrophage cell line following exposure to CPE.
3. To evaluate the serum cytokine response following intraperitoneal administration of sublethal quantities of CPE in the mouse model of human illness.
4. To evaluate the serum cytokine response following intragastric administration of sublethal quantities of CPE in the mouse model of human illness, which would more closely resemble the human illness, caused by CPE.
5. Following intragastric administration of sublethal quantities of CPE: to determine the organs which are transcriptionally active in producing mRNA coding for cytokines, to determine the time course of this

response, and to compare this response with the serum cytokine response in order to gain insight into the regulation of these responses.

6. To determine whether CPE is associated with altered levels of fecal bile acids in SIDS victims.
7. To determine whether neopterin, a product of activated macrophages, is present in higher levels in SIDS victims than it is in control infants in a small preliminary study.
8. To determine new areas of research which will prove helpful in the understanding of CPE intoxication.

The results of this work provide new insights into the response of the host following CPE intoxication. They also demonstrate similarities and differences between the responses observed and that seen following exposure to bacterial endotoxin (LPS) or the staphylococcal enterotoxins. The findings also suggest new avenues of research which will be helpful in further establishing the mechanism of pathogenicity of CPE.

CHAPTER 2
REVIEW OF THE LITERATURE

The Genus *Clostridium*

The clostridia are a diverse group of bacteria which are all anaerobic, rod-shaped and Gram positive. The oxygen tolerance of the organisms varies widely, with some being highly sensitive while others are able to grow on the surface of agar medium incubated aerobically. Morphology of these organisms ranges from short coccoid rods to long filamentous rods. Most are motile with peritrichous flagella. Members of this Genus are ubiquitous with species being found in soil, fresh water, and ocean sediments frequently in the spore state. Though some species are thermophiles or psychrophiles, the vast majority of species are mesophiles. The 1984 Bergey's Manual of Systemic Bacteriology provides detailed comprehensive descriptions of 85 species of clostridia, of which only a handful are pathogenic to humans (Holt, 1984). Despite the relatively few pathogenic species, there is a great diversity of pathology caused by clostridia. The Manual of Clinical Microbiology (Onderdonk and Allen, 1995) describes three categories of disease produced by clostridia. First, are the noninvasive diseases in which toxins cause pathology. Second, are the invasive diseases in which tissue is damaged by an infectious process. Third, are the purulent diseases, which are caused by a mixed population of bacteria including clostridia growing in a closed space, usually the peritoneum.

Clostridium perfringens

Clostridium perfringens is a sporeforming, Gram positive obligately anaerobic aerotolerant bacterium that causes considerable morbidity and mortality both in humans and in veterinary populations. This bacterium occurs singly or in pairs with large variations in cell dimensions and morphology. *C. perfringens* is encapsulated, nonmotile, and capable of producing a wide array of protein exotoxins responsible for its pathogenesis. *C. perfringens* is believed to be the most common cause of bacterial illness in domestic livestock, causing disease in chickens, cows, and sheep, among others (Songer, 1996). It is grouped into five types (A-E) based on the classic classification scheme of Hobbs (see table 1.1) (Rood and Cole, 1991; Songer, 1996). This typing system uses the production of four toxins (at least 13 are produced) as its basis. All five types were thought to produce the alpha toxin, which is also a phospholipase C, however this now appears not to be the case as strains of *C. perfringens* which do not produce this protein have been isolated (Lindsay, 1996). Typing of *C. perfringens* is performed with type-specific antisera. This procedure has been found to be 82% effective in determining a strain type (Murrell, 1989). Debate now exists as to whether this typing scheme should be revised taking into account the new molecular information and typing techniques developed regarding this organism (Lindsay, 1996). For those strains which defy typing by antisera, determination of bacteriocin sensitivity is an alternative method of evaluating strain type. In this procedure, blood agar media is plated with the strain to be typed. Bacteriocin is then added and zones of inhibition are then evaluated (Mahony et al., 1992). Another method to type *C. perfringens* is the evaluation of capsular

polysaccharide using instrumental techniques. This method as been suggested as a useful rapid technique for the evaluation of food-borne illness-producing isolates (Murrell, 1989).

Type A strains produce the appropriately named *C. perfringens* type A enterotoxin (CPE), which is responsible for a self-limiting diarrheal illness in man. CPE is also likely involved in the pathogenesis of two other illnesses. One form, which appears in malnourished infants and is characterized by colonization and invasion of intestinal tissue, produces a severe and sometimes deadly ulceration of the small intestinal mucosa. The other illness characterized by colonization with *C. perfringens* type A is the so-called infectious diarrhea syndrome in which usually elderly, institutionalized patients sustain a protracted colonization with *C. perfringens* leading to a diarrhea of long term duration for which antibiotic therapy is warranted (Larson and Borriello, 1988).

Table 2.1 Typing of *C. perfringens* by toxins produced (from Rood and Cole, 1991).

	Toxin produced				
C. perfringens type	α	β	ε	ι	δ
A	+++	-	-	-	-
B	+	++	+	-	+
C	+	++	-	-	+
D	+	-	++	-	-
E	+	-	-	+	-

Isolation and Sporulation in the Laboratory

Solid media for isolating *C. perfringens* is both selective and differential. Selectivity is conferred by the addition of antibiotics to which this organism is particularly resistant. Trypticase Sulfite Neomycin Agar (TSN), one of the most widely used isolation media, contains neomycin sulfate and polymyxin sulfate as selective agents. Differential ability is conferred by ferrous citrate and sulfites; during growth of *C. perfringens* sulfite is reduced and precipitates as iron sulfide, producing black colonies. Other media rely on the ability of the vast majority of strains of *C. perfringens* to produce phospholipase which give zones of hydrolysis in lecithin containing media.

In vitro sporulation is strain dependent with some strains sporulating abundantly and others proving difficult to induce to sporulate (Labbe, 1989). The most common media used to induce sporulation is the media of Duncan and Strong (Duncan and Strong, 1968). This liquid media consists of 0.4% yeast extract, 1.5% proteose peptone, 0.4% soluble starch, 0.1% sodium thioglycolate, and 1.0% sodium phosphate. Strains which prove difficult to induce to sporulate are sometimes assisted in this activity by the addition of raffinose or methylxanthines which may influence nucleic acid metabolism (Labbe, 1989).

C. perfringens Enterotoxin (CPE)

CPE is a 35 kD monomeric acidic protein (pI=4.3). It is considered a sporulation associated protein in that synthesis is upregulated during sporulation. It is also produced in small amounts under some vegetative growth conditions (Goldner et al., 1986; Rood

and Cole, 1991). *In vitro* experiments have shown that CPE acts by compromising the integrity of the cell membrane, disrupting electrolyte gradients and eventually leading to disruption of macromolecular synthesis (McClane, 1994). *In vivo* studies with animal models have shown fluid and electrolyte loss into the lumen of the small bowel following introduction of CPE into ileal loops (McClane, 1996; Lindsay, 1996). The toxin is most active in the ileum (the last portion of the small bowel), with lower activity in the jejunum and duodenum, and with no apparent activity in the colon likely due to its receptor mediated mode of action. These experiments also demonstrated histopathology of the small intestine manifested as a desquamation of the villous enterocytes. This is in contrast to other bacterial enterotoxins, exemplified by cholera toxin, that cause secretory diarrhea with no histopathological damage.

C. perfringens in Food-borne Illness

Though food-borne illness (FBI) presumptively caused by *C. perfringens* and its associated enterotoxin had been described in the 19th century (Klein, 1895), it was not until the 1940s that a definitive association was made between *C. perfringens* and this FBI (McClung, 1945). Since then, it has been recognized as the third most common cause of FBI in the USA. It is important to note that though we refer to *C. perfringens* food-borne illness as an intoxication, in the strictest sense it is not. In contrast to other intoxications in which preformed toxin is ingested, CPE toxicosis is the direct result of the consumption of $>10^7$ vegetative cells which, upon encountering the harsh environment of the gastrointestinal tract, are induced to sporulate. The sporulation process may be accompanied by the production of large amounts of CPE. This

enterotoxin is active in the small bowel and symptoms develop 8-24 hours after the consumption of contaminated food. The illness usually resolves spontaneously within 12-24 hours.

C. perfringens has a number of characteristics which contribute to its importance as a food-poisoning organism. Although classified as an anaerobe, *C. perfringens* exhibits a tolerance to elevated oxygen levels that is not seen with many other anaerobic bacteria. The relative aerotolerance allows growth in foods such as raw ground beef which has a relatively high oxidation/reduction potential (Eh). Spores of *C. perfringens* are ubiquitous, being found in virtually all soils examined. Vegetative cells of *C. perfringens* are also frequently found at low levels in the intestinal tract of many healthy animals including humans (Collee, 1974). As a consequence of its widespread presence, approximately 50% of raw and frozen meat products have been found to be contaminated with this organism (Labbe, 1989). Though *C. perfringens* may be frequently isolated from the intestinal tract of fish, seafood products are rarely involved in food poisoning. A major characteristic of *C. perfringens* that contributes to its prevalence as an agent of food-borne disease is its extraordinarily rapid doubling time (McClane, 1996). Under ideal laboratory conditions, *C. perfringens* can exhibit a generation time as rapid as seven minutes. This short generation time is likely a factor in making CPE food-borne illness so common.

Prevention of *C. perfringens* Food-borne Disease

The widespread distribution of *C. perfringens* spores and their resistance to destruction likely make it impossible to eliminate them from the food supply. Meat and

poultry products are the most common vehicles for this FBI because of the incidence of *C. perfringens* in such foods and its nutritional requirements (Labbe, 1989). It has been reported that improper cooling procedures or holding temperatures are responsible for 97% of reported FBI caused by this organism (McClane, 1996). To reduce the incidence of *C. perfringens* associated FBI, foods should be kept at temperatures not conducive to growth, either through storage at temperatures above 60^0C or below 15^0C following preparation. Especially important is not allowing foods to remain at *C. perfringens'* ideal growth temperature of $43\text{-}47^0$C. pH can also play a role in the prevention of *C. perfringens* growth. The pH range for growth of this organism is 5.0-8.3 but optimum growth occurs between 6.0 and 7.0.

Genetics of CPE Expression

CPE mRNA has an exceptionally long half-life of 58 minutes in sporulating cells (Labbe and Duncan, 1977). This is likely one reason for the very high expression of this protein during the sporulation process. The link of sporulation and CPE synthesis led to the hypothesis that CPE was a spore coat component (Frieben and Duncan, 1973). These workers used sporulation defective mutants of *C. perfringens* to study the production of CPE. Early stage spo0 sporulation mutants but not later stage spoV mutants proved defective in CPE production. Additional studies using immunolabeling and electron microscopy demonstrated that CPE was confined to the cytoplasm (Walker et al., 1975). Because of this work, the hypothesis that CPE was a structural component of the spore proved incorrect.

The complete CPE gene has been cloned and sequenced (Czeczulin et al., 1993) allowing much more detailed studies of the regulation of synthesis and genomic organization. The CPE mRNA has been determined to be transcribed as a monocistronic message with the origin of transcription located approximately 200 base pairs (BP) upstream of the CPE translation start site (Melville et al., 1997). The *Escherichia coli* transformant containing *cpe* was found to express low levels of CPE (apparently driven by a *C. perfringens* promoter) in amounts comparable to nonsporulating cultures of *C. perfringens*. Sporulating cultures of *C. perfringens* were found to produce vastly greater quantities of enterotoxin, suggesting that sporulation is not essential for CPE synthesis but does induce high-level synthesis (Czeczulin et al., 1993). The CPE gene has been shown to be contained on a mobile element, a transposon or lysogenized phage, which at least theoretically allows transfer of *cpe* between *C. perfringens* strains (Canard et al., 1992). The location on a mobile element likely explains the presence of *cpe* in both chromosomal and plasmid locations. Though the reason is not known, all food poisoning isolates examined have *cpe* integrated into the chromosome. Veterinary isolates, in contrast, frequently have a plasmid-borne *cpe* gene (Cornillot et al., 1995). The significance of the facts that *cpe* appears to be contained in a mobile element and that there are different locations for the gene in human and veterinary pathogenic strains is not clear. Some type C strains of *C. perfringens* have also been found to carry the *cpe* gene, without any evidence that they can cause FBI in humans (Lindsay, 1996). Further work examining the relationship between environmental, veterinary, and human associated strains will hopefully provide a more complete understanding of the source of food-borne disease-causing strains of *C. perfringens*. Also of interest is the finding that some *C.*

perfringens type E strains possess a silent form of the *cpe* gene. Whether this gene is an ancestral prototype of *cpe* or is the result of a later insertional event made possible by the presence of *cpe* on a mobile genetic element is the subject of much debate (Lindsay, 1996). This finding is especially interesting in light of the recent discoveries of other silent toxin genes in other species of clostridia (Lindsay, 1996). The relationship between silent clostridial genes, mobile genetic elements, and pathogenesis is a subject awaiting further research.

Symptoms of CPE Intoxication

Symptoms of the FBI associated with CPE include nausea, intestinal cramps, and a diarrhea which can range from mild to extreme (often described as explosive). Symptoms are apparent 8-24 hours after infection and usually spontaneously resolve within 24 hours of onset (Labbe, 1989). Fatalities are rare and those that occur are usually in the very young, elderly, or immunocompromised. *C. perfringens* type A and CPE have also been implicated in a more aggressive diarrheal illness resulting from a more persistent colonization and overgrowth of the bowel than that which occurs in the typical FBI. This illness most frequently occurs in the elderly, often in long term care facilities such as nursing homes, and often following antibiotic therapy. It has a more protracted course than the FBI, lasting on average 11 days (Larson and Boriello, 1988). Blood in the stool may be evident along with severe abdominal pain. This disease is in some ways similar to that caused by *C. difficile* following antibiotic therapy.

Human Feeding Studies

In human volunteer feeding studies, sublethal levels of CPE administered intragastrically, produce symptoms typical of *C. perfringens* type A induced FBI (Skjelkvale and Uemura, 1977) including diarrhea and abdominal cramps with pain. These studies revealed that administration of 100 ng toxin / g body weight is sufficient to induce this response. Other studies revealed that 10^8 vegetative cells administered orally were required to induce illness in human volunteers (Duncan and Strong, 1969).

Mechanisms of Action of CPE

CPE disrupts the integrity of plasma membranes of susceptible cells and it is through this activity that this enterotoxin exerts its cytotoxic effects (McClane, 1994). Detailed studies of the biochemistry of this receptor-mediated phenomenon have been conducted in the laboratory of McClane. As pronase pretreatment of brush border membranes reduced binding of CPE, the receptor(s) is/are thought to be proteinacious (Wnek and McClane, 1986). A 50 kDa protein from rabbit brush border membrane was identified as the putative enterotoxin receptor (Wnek and McClane, 1986). A similar protein was found to bind CPE on Vero cells (African green monkey kidney cells) and it was further found that the CPE-receptor complex aggregates to form a 160 kDa complex. By simple subtraction (35 kDa CPE, 50 kDa receptor), it was concluded that the additional binding substance was an approximately 70 kDa protein (Wiekowski et al., 1994). Detailed models of CPE binding, insertion, and conformational changes of CPE and receptor have been proposed to account for the experimental results using cloned portions and mutated forms of the CPE molecule and various biochemical techniques

(McClane, 1996). It should, however, be noted that recently another team of researchers has cloned and sequenced a 22 kDa protein from Vero cells which appears to be the CPE receptor (Katahira et al., 1997). The results of their study satisfied most of a molecular version of Koch's postulates. First, cells expressing the receptor were sensitive to the cytotoxic effect of CPE while cells not expressing this protein were resistant to this effect. Second, the 22 kDa receptor was found to render resistant cells sensitive to the enterotoxin when expressed in them. The only remaining work which must be done to make their work complete is the inactivation of their receptor in a sensitive cell line and the demonstration that this line is no longer sensitive to the effects of CPE. Results also showed that CPE could not assemble into a complex unless it interacted with this receptor. The rigor of the work conducted by Katahira is impressive, however it contradicts the results of McClane. How the two groups' findings can be reconciled remains to be determined.

After binding and insertion, CPE directly induces cell membrane permeability changes (McDonel and McClane, 1979; McClane, 1994). Ion-generated membrane polarity is compromised by these changes leading to inhibition of DNA, RNA, and protein synthesis (Hulkower et al., 1989). *In vivo* these changes are thought to impair cellular metabolism and eventually lead to cell death. As sensitive cells in the small bowel epithelium (in particular villous enterocytes) die, histopathological alterations occur with associated fluid and electrolyte loss into the lumen of the bowel with resultant diarrhea.

Support for the Use of the Mouse Model

Mice are the most common animal species used to study infectious processes. The reasons for this are grounded in both pragmatism and good science. Pragmatically, mice are inexpensive to purchase and maintain. Of greater importance is that inbred mouse strains allow the use of many genetically identical mice reducing the diversity of responses to infectious agents and their toxins seen in normal populations. This makes results more reliable and reproducible. Also of major benefit to those studying immune system activities, the genetic regions associated with immune function in these mouse strains are well characterized. Studies involving CPE have been conducted in a wide range of animals including sheep, calves, monkeys, rabbits and mice (Weiss et al., 1966; Tsai and Reiman, 1975; Lindsay and Dennison, 1986). Mice were found to elicit similar responses to humans upon exposure to CPE (Tsai and Reiman, 1975; Lindsay and Dennison, 1986).

Vaccine Possibilities

Vaccination of animals to induce resistance to infection has proven very successful. Vaccine strains are typically multivalent, containing killed cells, inactivated toxins, or both (Songer, 1996). Though vaccination in animals to prevent illness caused by *C. perfringens* is common, it is doubtful that such a strategy will be feasible in humans. First, many veterinary illnesses caused by *C. perfringens* are fatal, particularly in young animals, and thus contribute to large economic losses. In humans, in contrast, the vast majority of those affected experience a spontaneous recovery with no apparent long term sequelae. Second, there appears to be no long term immunity conferred by

infection in humans after CPE food-borne disease, despite the fact that there is detectable antibody to CPE for several weeks following exposure (Skjelkvale and Uemura, 1977; Mietzner et al., 1992). This may make design of a vaccine for humans difficult.

Antigens and Superantigens

Normal antigens from extracellular sources are taken up by Antigen Presenting Cells (APCs) and are degraded intracellularly to polypeptide fragments which are then presented in the context of MHC Class-II to T-Cells whose T-Cell receptor (TCR). If the binding region of the TCR possesses the proper protein sequence to interact strongly with the presented antigen, events are induced which lead to activation of the T-cell. Superantigens, in contrast, bind directly to MHC class II without uptake and subsequent processing. In the context of MHC class II, these proteins bind noncovalently to the TCR. This interaction occurs outside the normal binding groove of the TCR in a region designated the Vβ region (the variable region of the β chain of the TCR molecule). This interaction between MHC class II, superantigen, and TCR activates the T-cell to proliferate and produce cytokines just as a normal peptide antigen does. The difference between normal antigens and superantigens is the number of reacting cells. A normal peptide antigen can activate between 1 in 10^5 and 1 in 10^6 T-cells. Superantigens, on the other hand, can activate up to 1 in 4 T-cells. This is because of the limited number of Vβ regions expressed by an individual. For example, there are approximately 25 different Vβ regions in mice and approximately 60 in humans. As each superantigen normally binds to more than one Vβ region it is obvious that a large fraction of the T-cell

population can become activated. Activating such a substantial number of T-cells can have extremely harmful effects. Normally when an individuals' immune system is challenged a balance is attained generating a vigorous immune response which will clear the invader but not one which is so great that harm to the organism results. In the short term, the signaling molecules of the immune system, the cytokines, can induce harm to the organism. Cytokines in excessive amounts have been implicated in a host of pathologic states including septic shock due to systemic exposure to endotoxin and the toxic shock syndrome from interaction with a staphylococcal exotoxin, toxic shock syndrome toxin-1 (TSST-1) (Miethke et al., 1993). In limited amounts these protein mediators perform valuable functions. They serve as chemotaxins, movement inhibitory factors, and activation signals for the mobile phagocytic cells of the immune system (Thomson, 1998; Mire-Sluis and Thorpe, 1998). They also induce maturation of cells leading to specific humoral immunity to invading pathogens (Thomson, 1998; Mire-Sluis and Thorpe, 1998). As such they are indispensable for healthy immune function (Thomson, 1998; Mire-Sluis and Thorpe, 1998). The D-galactosamine (D-gal) sensitized mouse model is frequently used to study the harmful (lethal) effects of an overproduction of cytokines (Meithke et al., 1993; Freudenberg et al., 1986). This system is used because mice are normally much more resistant to the effects of bacterial products than man. D-gal sensitization engenders a response in mice which renders them approximately as sensitive as man to the effects of these toxic substances.

CPE as a Superantigen

Recent work suggested that CPE was a superantigen selectively expanding the population of T-Cells bearing the TCR Vβs 6.9 and 22 greatly and Vβs 24, 21, 18, 5, and 6.1-6.5 to a lesser extent (Bowness et al., 1992). These results were exciting as they suggested that much of the observed pathology could be caused by mechanisms similar to those of the staphylococcal and streptococcal superantigenic enterotoxins. Though the work concluding that CPE was a superantigen proved incorrect (Krakauer et al., 1997; Wallace et al., 1999), it is possible that another contaminating protein produced by *C. perfringens* is in fact a superantigen. To date no work has been conducted to test this hypothesis.

Cytokines and the Inflammatory Immune Response

Host proteins, specifically cytokines, control the functions that lead to non-specific host defenses and specific immunity following challenge by foreign substances. Recent research has examined the role of cytokines in the observed host response to bacterial toxins. As this work has progressed, it has become clear that many of these bacterial products which were originally studied to elucidate their cytopathic effects possess the ability to modulate the host defense systems. It is now recognized that this interaction, through the generation of pro- and anti-inflammatory cytokines, is frequently as important as their direct effects in the induction of host pathology. Bacteria and their toxins trigger the synthesis and release of cytokines, which can act in autocrine, paracrine, or endocrine fashion leading to the generation of an immune response. Once initiated, these responses can have deleterious as well as protective aspects. Besides

leading to clearance of infection and protection from future infection, there are negative aspects to these responses. Once initiated, the production of these potent mediators can generate a cascade of interactive cytokines; the host's response is amplified as cytokines induce synthesis of other cytokines. Taken to extreme, these responses elicit septic shock in response to Gram negative bacterial lipopolysaccharide or toxic shock in response to staphylococcal toxic shock syndrome toxin-1 (TSST-1), both of which can be lethal to the organism generating the response. Most times there is exposure to bacterial products these deleterious processes do not occur, as there are potent mechanisms for depressing cytokine networks as well. Examples of this regulatory pattern include: tumor necrosis factor-α (TNF) inducing susceptible cells to shed their TNF receptors and making these cells non-responsive to further stimulation by this cytokine, and interleukin-1 (IL-1) inducing synthesis of IL-1 receptor antagonist thus blocking the receptors for IL-1 and making susceptible cells anergic to the effects of additional IL-1 (Cavaillon, 1994). This is of critical importance, as the balance of positive and negative effector molecules appears critical in the outcome of the detrimental effects of inflammation such as shock. Several proinflammatory cytokines also act to induce anti-inflammatory cytokines thus regulating the intensity of the immune reaction.

The proinflammatory cytokines have been extensively studied in relation to their central role in the immune response to bacterial toxins and various infections: interferon-γ (IFN-γ), interleukins-1, -2, and -6 (IL-1, -2, -6), and TNF have been extensively studied (See Table 1 for a summary).

Briefly, IFN-γ is produced by activated T-cells and NK cells and is one of the central components of the pro-inflammatory response. Known functions of this cytokine

include upregulation of MHC Class-II expression, and activation of macrophages. As is the case with many of the proinflammatory cytokines, IFN-γ also induces anti-inflammatory cytokines which serve to attenuate the immune response and limit self-induced pathology.

IL-1 is a cytokine which is proving to be central in the outcome of infectious disease, particularly those of a bacterial nature. It is produced primarily by macrophages, monocytes, and Küpffer cells. Local effects of IL-1 are neutrophile chemotaxis and release of lipid-derived mediators. Systemic effects include fever, hypotension, induction of cytokine synthesis, and the induction of the acute phase response (Dinarello, 1992).

IL-2 is a product of activated T-cells only and is thus an excellent maker of T-cell function. The major function of IL-2 is the induction of a series of steps leading to the clonal expansion of antigen specific T-cells.

The principal cytokine stimulating the acute phase response during inflammation appears to be IL-6. It is produced primarily by macrophages and monocytes and also by cytokine stimulated epithelial and endothelial cells. This cytokine is of additional interest because of its role as an antagonist of TNF-α. In some experimental models using the D-gal sensitized murine model of septic shock a protective role against TNF-α mediated mortality was shown for IL-6 (Barton and Jackson, 1993). This effect may be mediated through the acute phase proteins as turpentine (a known inducer of the acute phase response) pretreatment of D-gal sensitized mice offered protection from lethal shock. Though IL-6 is classified as a proinflammatory cytokine it is becoming increasingly clear that this cytokine serves an anti-inflammatory function as well.

TNF-α appears to be the central molecule inducing shock in the D-gal sensitized murine model of septic shock (Tracey and Cerami, 1993) satisfying a Koch's postulates test for a cause and effect relationship with this disease state. TNF is produced during septic shock, causes the syndrome when given to uninfected animals, and when neutralized in septic animals prevents pathology. TNF is the only cytokine so far determined to cause the entire spectrum of symptoms of septic shock including hemodynamic, metabolic, and pathological sequelae (Tracey and Cerami, 1993). Local effects of TNF during infection are of great benefit. Of particular importance is its role in the promotion of margination of leukocytes at the site of inflammation (Bemelmans et al., 1996).

C. perfringens and the Sudden Infant Death Syndrome

The sudden infant death syndrome (SIDS) is defined as the "sudden death of an infant under one year of age that remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene and the review of the clinical history" by the National Institute of Child Health and Development (Willinger, 1989). It remains the leading cause of post-neonatal mortality in the United States despite recent large declines, from approximately 2/1000 live births a decade ago to approximately 1/1000 live births today (Scott et al., 1998). These declines are probably due to the campaign to encourage parents to avoid the prone sleeping position for their infants, which is a major risk factor for SIDS (Scott et al., 1998). Virtually every area which has been investigated in relation to SIDS has been found to have differences with control infants, whether in gross anatomy, neuroanatomy, biochemistry,

or microbiology. From this body of research it is beginning to appear likely that there are large numbers of abnormalities in SIDS infants and that many of them may combine to induce vulnerability in an infant. One of the most common features of SIDS infants is that >85% were ill in the two weeks prior to death and indeed findings on autopsy do provide some support for an infectious process occurring in SIDS (Krous, 1984; Beckwith, 1988). The Sudden Infant Death Syndrome has also been associated with *C. perfringens* and with CPE. 50-80% of SIDS victims and less than 10% of control infants have demonstrated elevated levels of *C. perfringens* spores and/or enterotoxin in their feces leading some to propose a role for CPE in this syndrome (Lindsay, 1996). This finding that the vast majority of SIDS infants have elevated levels of *C. perfringens* spores and CPE in their bowel is of interest and suggests that CPE may act as a final trigger in SIDS for an infant made vulnerable in a variety of ways (Wilkinson, 1991; Lindsay et al., 1993). IL-6 levels have been found to be elevated in the cerebrospinal fluid of SIDS infants which provides further support that there is an immune reaction occuring before death from SIDS (Vege et al., 1995). Agents which activate cells of the monocyte/macrophage lineage must be considered as inducers of this increased IL-6 level.

Table 2.2 Sources and effects of the major proinflammatory cytokines.

Cytokine	Major Source	Effect
IFN-γ	T-cells and NK cells	-Antiviral and antiprotazoal activities -Increased expression of MHC class II on macrophages and T-cells -Activates macrophage tumoricidal activity -Induces formation and release of TNF by macrophages -Depending on environment either increases or decreases T suppressor cell activity
IL-1α	Monocytes, macrophages, Küpffer cells, glial cells	-Initiate acute phase reaction, fever induction -Through nitric oxide induces hypotension -Induces nausea and vomiting -Inhibits IL-6 production
IL-2	Activated T-helper cells	-Clonal expansion of antigen specific T-cells -Induces capillary leakage
IL-6	Monocytes, macrophages, cytokine stimulated endothelium and epithelium	-Primary cytokine involved in acute phase induction -Induces proliferation of pluripotential hematopoietic progenitor cells (*in vitro*) -Possibly involved in nerve cell function -Stimulates secretion of adrenocorticotrophic hormone
TNF-α	Monocytes, macrophages, Küpffer cells	-Primes the immune response -Induces shock, tissue injury, hypotension, fever, gastrointestinal ischemia, capillary leakage, and anorexia -Increased phagocytic activity of polymorphonuclear leukocytes -Induces transmigration and chemotaxis of monocytes

CHAPTER 3
AN INVESTIGATION OF THE PROPERTIES OF CLOSTRIDIUM PERFRINGENS TYPE-A ENTEROTOXIN USING D-GALACTOSAMINE AND CYCLOSPORIN-A

Introduction

In the investigation of the interaction of bacterial products and the host, agents which potentiate or abrogate the responses of certain components of the immune system are of great value. These agents were used to investigate the claim (Bowness et al., 1992) that the *Clostridium perfringens* type A enterotoxin (CPE) is a superantigen reacting strongly with Vβ 6.9 and 22 and weakly with Vβs 24, 21, 18, 5, and 6.1-6.5. Were this claim true, CPE would interact with the immune system in a manner similar to that seen with the staphylococcal enterotoxins.

This series of experiments established the mouse lethal dose (MLD, which is a measure of biological activity) for intraperitoneal (IP) and intragastric (IG) administration of a preparation of CPE. Following this determination D-galactosamine (2-amino-2-deoxy-D-galactose) (D-gal) sensitization was attempted followed by CPE administration. D-gal renders mice exquisitely sensitive to a range of bacterial toxins including Gram-negative bacterial lipopolysaccharide (LPS) and the staphylococcal superantigenic enterotoxins (Meithke et al., 1993; Freudenberg et al., 1986). The effects of D-gal on the lethal activity of LPS are an increase in sensitivity of up to 100,000 fold (Galanos et al., 1979). D-gal in investigations of the lethal effects of superantigens is of even greater importance as an investigatory tool. Normally mice are much more resistant to the

effects of superantigens than humans and do not succumb to the shock seen in humans unless first sensitized with this agent (Miethke et al., 1993). D-gal is a hepatotoxic chemical which, within 30 min of administration, induces the production of large amounts of UDP-galactosamine derivatives (Galanos et al., 1979). This depletes hepatic UTP which results in the cessation of synthesis of macromolecules. The effects of D-gal were thought to be confined to the liver with no damage produced in other organ systems but recent work indicates extensive apoptosis in the medullary pyramids in the kidneys of D-gal sensitized, LPS treated mice (Morikana et al., 1996). D-gal alone is not toxic and levels of up to 1 g D-gal / kg body weight do not induce any signs of injury (Galanos et al., 1979).

Cyclosporin-A (CyA) is another immunomodulatory compound which is of great use in the elucidation of the components of the immune system involved in host reactions. CyA is a fungal polypeptide with great immunosuppressive ability. It is used to prevent host rejection of transplanted organs in humans. Although its mechanism of action is not completely understood, it is generally accepted that CyA mediates its effects by interfering with T-cell activity, specifically the generation of cytokines (Nguyen et al., 1990; Meithke et al., 1992). Were T-cell mediated events implicated in the lethal events following CPE exposure, one would expect to see a protective effect from the administration of CyA before CPE challenge.

Materials and Methods

Reagents

CPE was obtained as a purified, freeze dried powder from Dr. Bruce McClane (University of Pittsburgh) on dry ice. Purity was determined through SDS-polyacrylamide gel electrophoresis with only one band visible following staining with Coomassie blue. Toxin was frozen in this form at -70^0C until use. Before administration toxin was resuspended in phosphate buffered saline-Tween 20 (PBS-Tw)(0.15 M NaCl, 0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.2% Tween 20, pH 7.2). Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. From this determination, the solution was diluted to a final concentration of 1 μg protein/μl diluent with PBS-Tw.

D-gal was obtained from Sigma while CyA was a generous gift of Novartis (formerly Sandoz, East Hanover, NJ). For D-gal sensitization studies mice were injected intraperitoneally (IP) with various concentrations of CPE with or without four hour pretreatment with 40 mg D-gal in 100μl PBS. D-gal in PBS was used as a control. For CyA studies, mice were injected IP with 400 μg CyA in PBS 4 hr before IP injection of CPE. CyA in PBS was used as a control.

Mice

16.5 $\pm$ 1.0 g male Swiss Webster (SW) mice or BALB/C mice for these studies were obtained through the Department of Animal Resources of the University of Florida from Harlan-Sprague Dawley (Indianapolis, IN). Department of Animal Resources personnel order, deliver, and care for the animals within the Food Science and Human

Nutrition Department Animal Facility as prescribed by the university of Florida IACUC. Animals were maintained on a 12 hr/12 hr light/dark cycle at 25^0C, six per cage and were examined daily by Animal Resources personnel who also change bedding and provide food *ad libitum*. Studies were performed in as humane a manner as possible with attention to preventing suffering on the part of the animals. Approval for all animal studies was granted by the University of Florida Animal Care and Use Committee (IACUC).

Toxin Administration and Mouse Lethal Dose (MLD)

For intraperitoneal (IP) administration one researcher held the mice in an inverted, fully extended position while another researcher measured toxin volume and performed the injection into the peritoneal cavity. Animals were administered CPE IP in the left side of the peritoneal cavity using a 1 ml Tuberculin syringe with a 26 gauge 10mm length needle. Injection volume, regardless of toxin amount was 100 µl. For intragastric (IG) administration (which was necessary to determine for work detailed in later chapters) mice were held in an inverted, fully extended position. Another researcher measured toxin volume (again 100µl) and used a Popper gastric ball head needle affixed to a 1 ml Tuberculin syringe to administer CPE directly into the stomach. After administration of toxin, animals were returned to their cages and monitored every 15 min. Within each experiment, mice were randomly chosen for group assignment. The time to death method of Spearman-Kerber (Zar, 1984) was used to determine the IP and IG mouse lethal doses (MLD) for CPE.

Results

Mice challenged either IP or IG with CPE in doses sufficient to cause an observable response exhibited the same symptoms. The first signs were an accelerated heart rate, a convex arched back, ruffled fur, opaque brownish eyes (vs. the normal bright red), immobility, accelerated shallow respiration, loss of appetite, and the social behavior of huddling together. Mice administered non-lethal doses of CPE (0.1 μg/g for SW mice challenged IP or 1.0 μg/g for those challenged IG) recover from all symptoms within 6-8 hours. Mice administered higher non-lethal doses (0.5 μg/g IP or 5 μg/g IG) recovered between 12-16 hr after challenge. No long-term effects were observed for the 48 hours following recovery.

Figures 3.1 A and 3.1 B show that the MLD for SW mice administered CPE IP is approximately 0.75 μg CPE / g body weight while the IG MLD is approximately ten fold higher at 7.5 μg CPE / g body weight.

Following determination of the MLD for CPE, experiments examining the effect of immunomodulatory agents on CPE toxicity were conducted. Data shown in Table 3.1 demonstrates that D-gal had no sensitizing effect for SW mice exposed IP to CPE. CyA had no protective effect in the Swiss White model system as shown in Table 3.2.

Following these results experiments to determine the MLD of CPE in BALB/c mice were conducted. These experiments established an MLD of approximately 0.5 μg CPE / g body weight for IP administration (data not shown). The D-gal and CyA experiments were repeated using the BALB/c mouse model system. Again, no sensitization was demonstrated following D-gal pretreatment and no protection was

evident following pretreatment with CyA as is shown in Tables 3.3 and 3.4. Additionally CyA did not increase the time to death of mice administered lethal doses of CPE over control mice administered the same dose of toxin without CyA pretreatment. Results from experiments performed by Torres working with Johnson, Wallace, and Lindsay (Lindsay, 1996) showed that CPE did not behave as a superantigen with regard to its interaction with major histocompatibility complex (MHC). Using RAJI cells, which are a high MHC class II producing cell line, these researchers were not able to demonstrate any competition between radiolabeled CPE and a panel of known superantigens including the staphylococcal enterotoxins. In a second experiment they used a murine L-cell line which is a mouse cell line transfected to express human MHC class II (and mouse MHC class I). In this experiment there was no increased binding to the L-cell line when compared to an untransfected control cell line. Both of these results are in conflict with the results which were expected if CPE did exhibit superantigenic properties.

Discussion

When this series of experiments was begun using SW mice, we were operating under the assumption that CPE was a superantigen (Bowness, 1992). After examining the results obtained in the D-gal sensitization and CyA protection studies, explanations that were still consistent with CPE being a superantigen were entertained. After further review of studies in mouse genetics, it was found that the SW strain of mice were missing part of the T-cell receptor (TCR) locus (Pullen et al., 1990). It then seemed possible that this strain of mice was missing the elements that would allow CPE to behave as a superantigen. It has been hypothesized that the deletion of portions of the TCR locus is

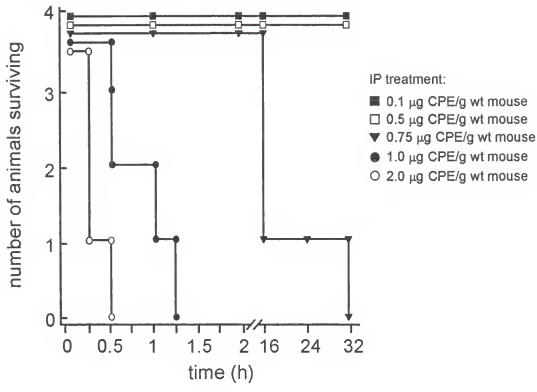


Figure 3.1 Determination of Mouse Lethal Dose (MLD) for CPE Administered IP.

protective in mice allowing them to resist the effects of pathogens which possess superantigenic proteins (Pullen et al., 1990). BALB/c mice were found to possess the complete TCR locus and were thus chosen as experimental animals to repeat the D-gal and CyA studies. Initial results were encouraging with BALB/c mice exhibiting greater sensitivity to CPE than SW mice (MLD of 0.15 μg CPE / g mouse weight for BALB/c

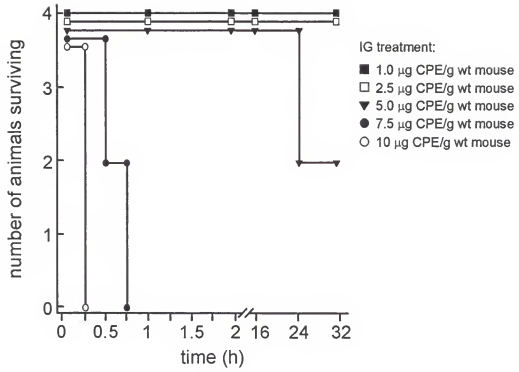


Figure 3.2 Determination of Mouse Lethal Dose (MLD) for CPE Administered IG.

mice versus 0.5 μg CPE /g mouse weight for SW mice). Repeating the procedures using BALB/c mice however showed no sensitization with D-gal and no protection with CyA pretreatment. Other bacterial superantigens that have proved to be superantigenic in humans have proved to possess the same properties in mice. The possibility that CPE was different and only behaved as a superantigen in humans but not in mice was considered. Dr. Howard Johnson and Dr. Barbara Torres (University of Florida, Department of Microbiology and Cell Science) were then consulted based on their

Table 3.1. Effect of D-galactosamine (D-gal) on CPE sensitivity for Swiss Webster mice.

Treatment	Lethality (dead/total)
PBS	0/6
40 mg D-gal	0/6
750 ng CPE	**6/6**
375 ng CPE	0/6
188 ng CPE	0/6
750 ng CPE + 40 mg D-gal	**6/6**
375 ng CPE + 40 mg D-gal	0/6
188 ng CPE + 40 mg D-gal	0/6
10 μg LPS	0/6
10 μg LPS + 40 mg D-gal	6/6

Note: CPE weights are given in ng CPE / g mouse weight.

expertise in working with superantigens. Using tissue culture based studies to evaluate CPE interaction with human MHC class II, Johnson and Torres concluded that CPE did not behave as a superantigen in humans and the sensitization and protection studies had determined that CPE did not behave as a superantigen in the mouse. The above outlined results indicate that, contrary to the claim that CPE is a superantigen, it most assuredly is

Table 3.2 Effect of Cyclosporin A (CyA) on CPE sensitivity for Swiss Webster mice.

Treatment	Lethality (dead/total)
PBS	0/6
400 μg CyA	0/6
750 ng CPE	**6/6**
375 ng CPE	0/6
188 ng CPE	0/6
750 ng CPE + 400 μg CyA	**6/6**
375 ng CPE + 400 μg CyA	0/6
188 ng CPE + 400 μg CyA	0/6
10 μg LPS	0/6

Note: CPE weights are given in ng CPE / g mouse weight.

not. *In vitro* studies did, however, indicate that CPE activated cells of a macrophage lineage (Wallace et al., 1999). This result, combined with the symptoms of *C. perfringens* type A food-borne illness in both humans and in the mouse model, led us to believe that CPE may interact with the immune system to induce proinflammatory cytokines. As such, we undertook a series of experiments examining the serum cytokines

Table 3.3. Effect of D-galactosamine (D-gal) on CPE sensitivity for BALB/c mice.

Treatment	Lethality (dead/total)
PBS	0/6
40 mg D-gal	0/6
500 ng CPE	**6/6**
250 ng CPE	**2/6**
125 ng CPE	0/6
500 ng CPE + 40 mg D-gal	**6/6**
250 ng CPE + 40 mg D-gal	**1/6**
125 ng CPE + 40 mg D-gal	0/6
10 μg LPS	0/6
10 μg LPS + 40 mg D-gal	**6/6**

Note: CPE weights are given in ng CPE / g mouse weight.

generated following IP and IG challenge of mice with CPE using the SW model system as well as examining the *in vitro* cytokine response of a macrophage cell line following incubation with CPE. The results of these experiments are described in Chapter 4.

Table 3.4. Effect of Cyclosporin A (CyA) on CPE sensitivity for BALB/c mice.

Treatment	Lethality (dead/total)
PBS	0/6
400 μg CyA	0/6
500 ng CPE	**6/6**
250 ng CPE	0/6
125 ng CPE	0/6
500 ng CPE + 400 μg CyA	**6/6**
250 ng CPE + 400 μg CyA	**1/6**
125 ng CPE + 400 μg CyA	0/6
10 μg LPS	0/6

Note: CPE weights are given in ng CPE / g mouse weight.

CHAPTER 4

SERUM CYTOKINE LEVELS IN SWISS WEBSTER MICE EXPOSED IP AND IG TO CPE AND IN VITRO CYTOKINE PRODUCTION OF A MACROPHAGE CELL LINE EXPOSED TO CPE

Introduction

Data from studies described in Chapter 3 determined that CPE was not a superantigen; however, CPE is nonetheless a bacterial exotoxin and many such products have been found to induce an immune response (Flegel et al., 1991; Misfeldt et al., 1990; Bhakdi et al., 1990). Previous work (Mach and Lindsay, 1997) had indicated that *in vitro*, the murine macrophage cell line J774A.1 was activated to produce nitric oxide following exposure to CPE. Their work also documented a short-term (12-24 hour) mitogenic effect and a longer term (48 hours and greater) lethal effect. Lindsay, Torres, and Johnson had also documented a mitogenic effect in human peripheral blood mononuclear cells (PBMC) exposed to CPE (Lindsay, 1996; Wallace et al., 1999). These results suggested that there was an *in vitro* production of cytokines by the macrophage cell line previously used to demonstrate activation by CPE. The results also suggested an activation of cells of the macrophage/monocyte lineage would likely occur after *in vivo* challenge by CPE with concomitant production of cytokines. This chapter documents results showing that, indeed, the J774A.1 cell line did produce cytokines following exposure to CPE, and mice challenged with CPE IG and IP did exhibit a cytokine response.

Materials and Methods

Reagents

Freeze dried CPE obtained from Dr. Bruce McClane, reconstituted, handled, and administered as described in Chapter 3. Reconstitution was performed the day of administration as CPE in solution loses biological activity even when stored at -70^{0}C. Biological activity was confirmed using the Vero cell cytotoxicity assay (Wallace et al., 1999). As before, protein concentration was determined by the method of Lowry et al., (1951) with bovine serum albumin as a standard. From this determination the solution was diluted to a final concentration of 1 μg protein/μl diluent with PBS-Tw.

Tissue Culture

J774A.1 (ATCC TIB 67) macrophage cell line cultures were incubated as adherent monolayers in a humidified incubator in 5% CO_2 at 37^{0}C using Sarstedt 75 cm^2 flasks and modified Dulbecco's media (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1 mM sodium pyruvate. When flasks were >80% confluent, cells were removed by gentle scraping, and transferred to sterile conical tubes and centrifuged at 200 x *g* for 5 min. The supernatant was removed and pellets combined in 10 ml of fresh DMEM. Approximately 10^5 cells were seeded/well, as determined by hemicytometer counts, into Sarstedt 24 well cluster dishes and incubated for 24 h. During this 24 h incubation/resuscitation, cell numbers do not change significantly from the initial inoculation. The medium was then removed, and the cells washed twice with 15 mM PBS, pH 7.0 to remove any residual FBS. Cells were incubated in 0.4 ml Earl's

balanced salts containing 0.28 mM phenol red (EBS-PR) without FBS, amino acids or vitamins.

Measurement of Cytokine Levels *in vitro*

J774A.1 cells were incubated with 10 μg CPE/well and examined after 24 h. Supernatants were collected and levels of cytokines IFN-γ, TNF-α, IL-1α, IL-2, and IL-6 were determined using commercially available ELISA kits obtained from Endogen (Cambridge, MA). Internal controls were always run with each cytokine test, the sensitivity of each test being <15 pg/ml. Results are means ± SE (n=6) based on 10^5 cells/well. Cytokine levels were normalized to control wells run with all reagents except cytokine. Significance was calculated using the Student's *t*-test.

Mice

16.5 ± 1.0 g male Swiss Webster (SW) mice for these studies were obtained through the Department of Animal Resources of the University of Florida from Harlan-Sprague Dawley. Department of Animal Resources personnel ordered, treated, and cared for these animals as described in Chapter 3.

Toxin Administration

Mice were administered toxin essentially as described in Chapter 3. Within each experiment, mice were randomly chosen for group assignment. Mice were given 0.6 μg CPE/g body weight in IP injection, or 4.5 μg CPE/g body weight in IG administration.

These toxin amounts were slightly below 0.5 MLD which has been found to elicit typical pathophysiological symptoms as described in Chapter 3 while not risking the possibility of death.

Blood Collection and Serum Isolation

At the times indicated, mice were anesthetized using chloroform. Following anesthesia whole blood was collected. Blood samples were allowed to clot at 4^0C for 2 h, and centrifuged at 200 x g to obtain serum. Serum samples were stored at -70^0C until cytokine determination.

In vivo Cytokine Determination

Levels of cytokines IFN-γ, TNF-α, IL-1α, IL-2, and IL-6 were determined using commercially available ELISA kits obtained from Endogen (Cambridge, MA). Internal controls were always run with each cytokine test, the sensitivity of each test being <15 pg/ml. Cytokine levels were normalized to control wells run with all reagents except cytokine. Data in ng/ml are reported as means $\pm$ SE (if greater than 10% of the mean) in Figs 4.1-4.10.

Results

Cytokine levels *in vitro*

CPE was found to induce the synthesis of 5.8 $\pm$ 0.02 μg/ml IFN-γ; 4.5 $\pm$ 0.01 μg/ml TNF-α; 1.3 $\pm$ 0.03 μg/ml IL-1α; and 3.0 μg/ml IL-6 in the macrophage cell line J774A.1 following incubation with CPE. As expected no IL-2 was produced.

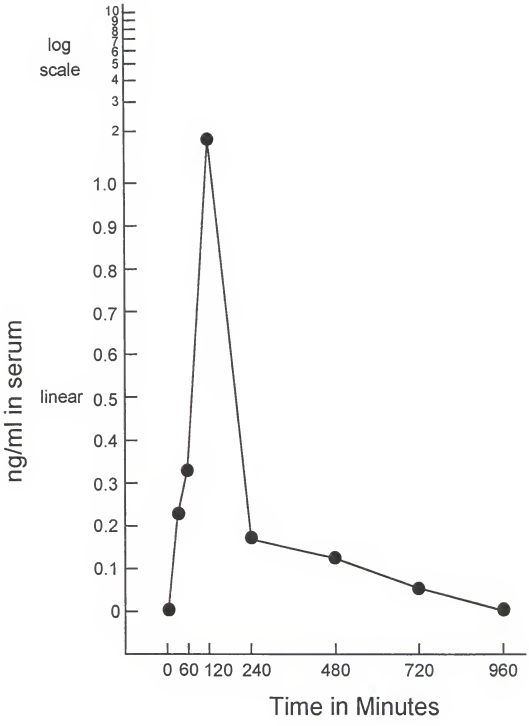


Figure 4.1 Serum levels of IL-1α following IP administration of CPE.

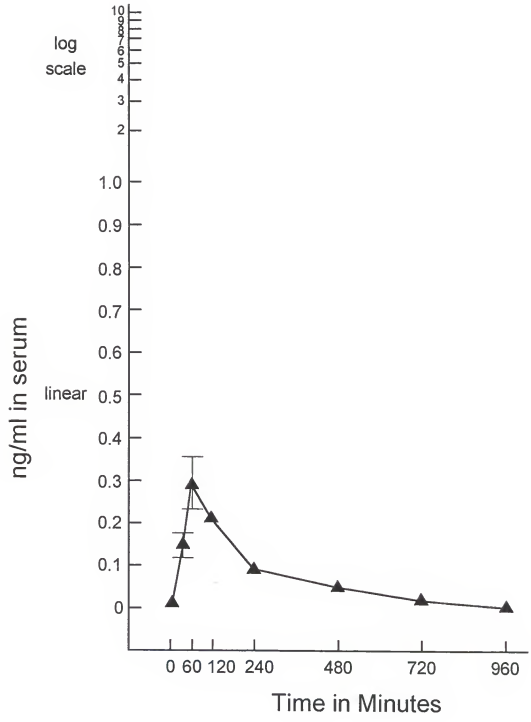


Figure 4.2 Serum levels of IL-2 following IP administration of CPE.

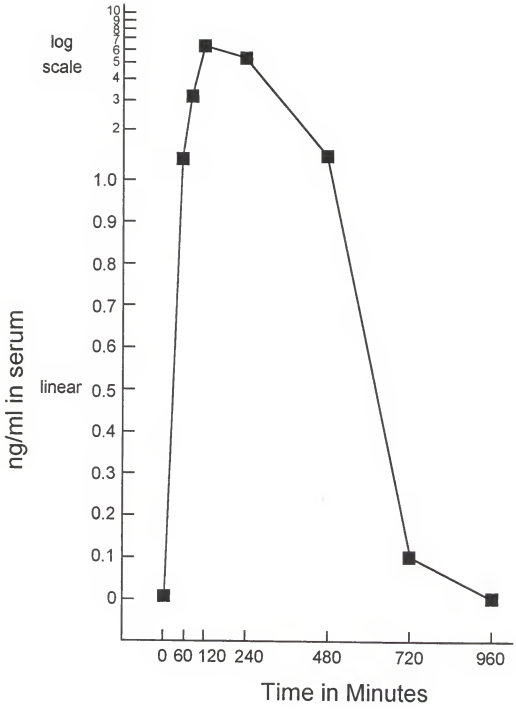


Figure 4.3 Serum levels of IL-6 following IP administration of CPE.

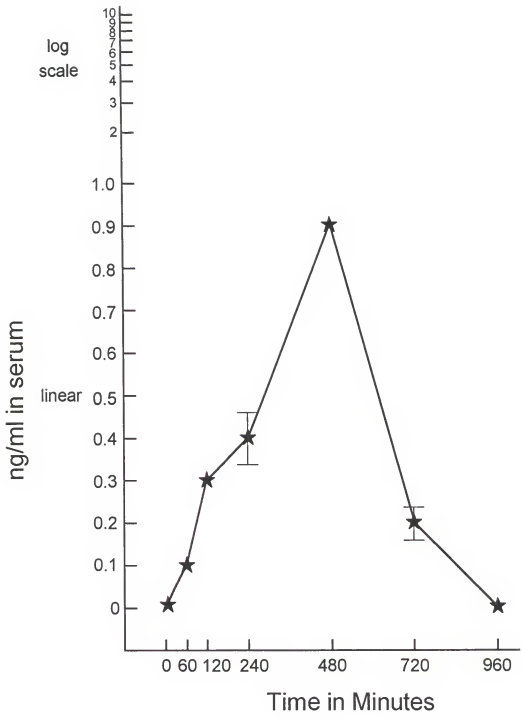


Figure 4.4 Serum levels of IFN-γ following IP administration of CPE.

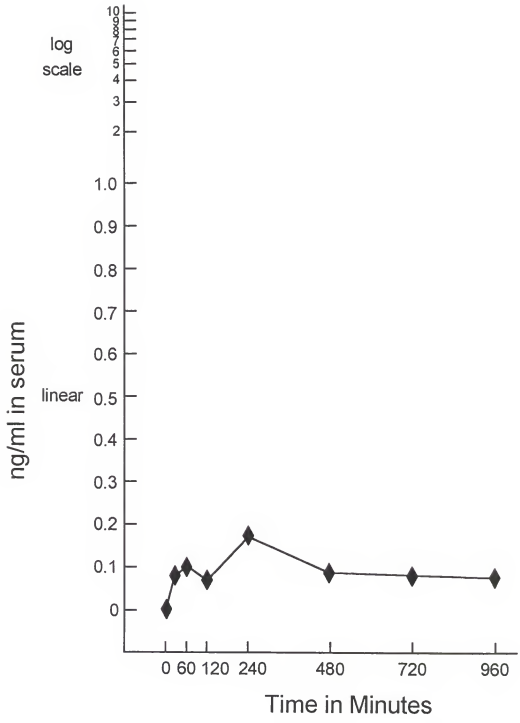


Figure 4.5 Serum levels of TNF-α following IP administration of CPE.

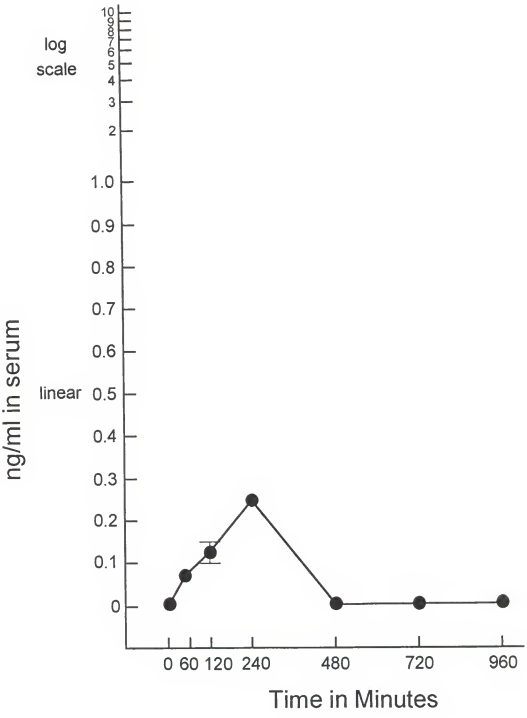


Figure 4.6 Serum levels of IL-1α following IG administration of CPE.

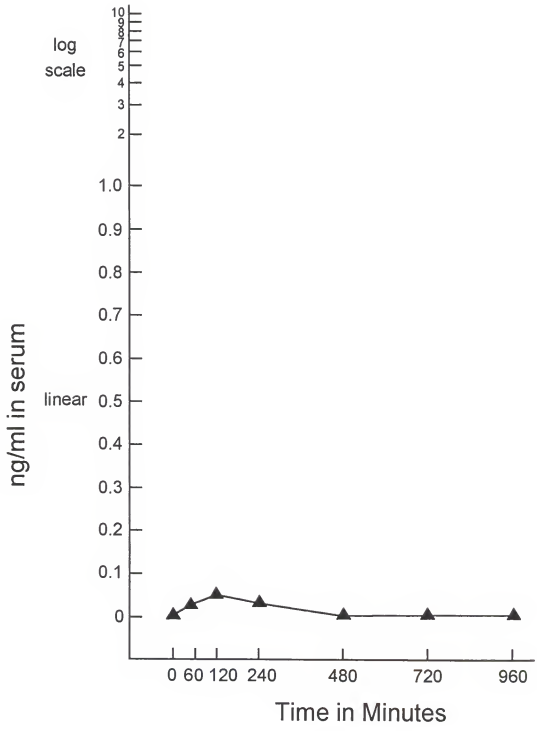


Figure 4.7 Serum levels of IL-2 following IG administration of CPE.

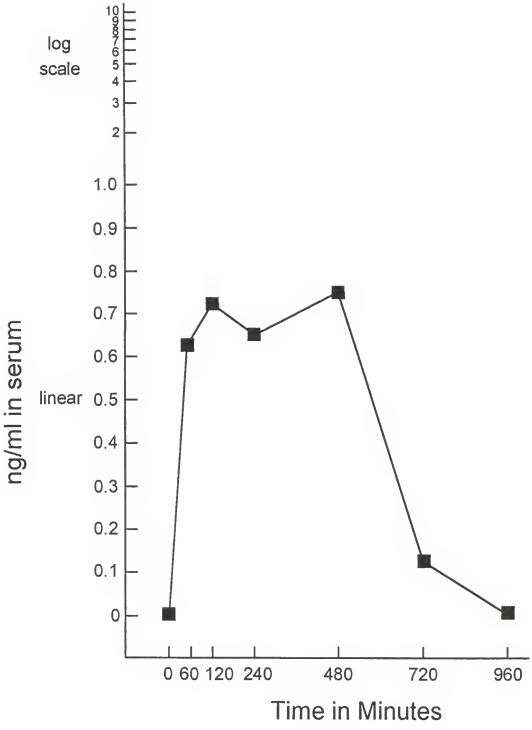


Figure 4.8 Serum levels of IL-6 following IG administration of CPE.

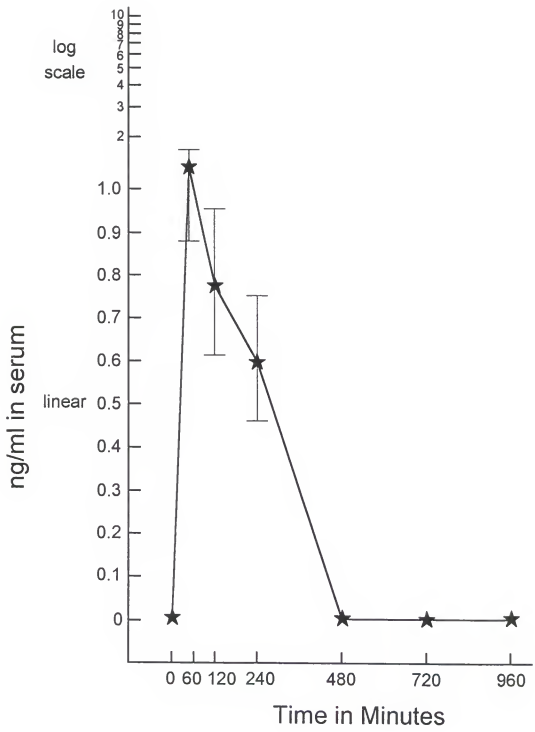


Figure 4.9 Serum levels of IFN-γ following IG administration of CPE.

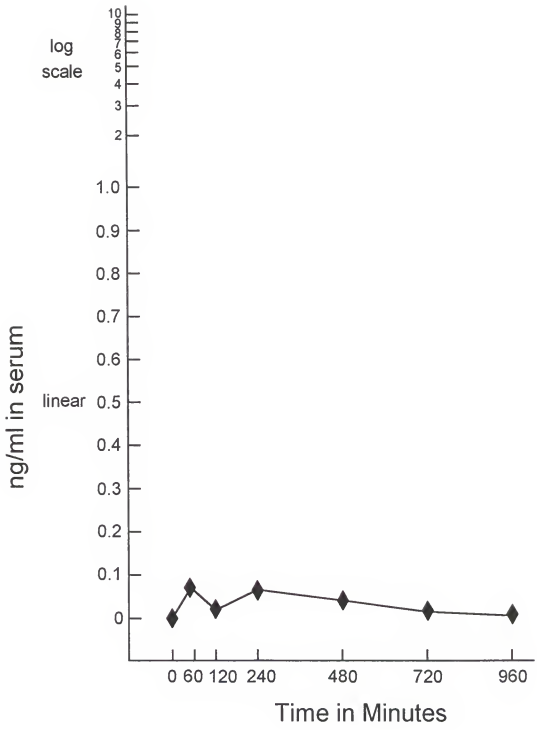


Figure 4.10 Serum levels of TNF-α following IG administration of CPE.

Cytokine levels *in vivo*

Figures 4.1-4.10 show the kinetics of cytokine induction after IP (Figs 4.1-4.5) and IG (Figs 4.6-4.10) administration of slightly less than 0.5 MLD. With one exception, all cytokine levels markedly increased within 0.5 h and peaked between 1-2 h. Maximum levels of IFN-γ were maintained for 8 h for IP administration of toxin and 4 h for IG administration. Levels of IL-6 were maintained for 8 h, while IL-1α, and the very low levels of IL-2 peaked and fell to nearly baseline levels within 3-4 h. After an initial increase, TNF-α levels fluctuated with a further increase after 4 h. This phenomenon was consistently detected in all animals without regard to route of administration (IP or IG). All cytokine levels were very low after 12 h and had returned to baseline levels by 16 h. Challenge by IP administration of CPE induced nearly 10 fold higher levels of IL-1α and IL-6, and 3 fold higher levels of TNF-α and IL-2 than by IG administration. Peak IFN-γ levels were similar regardless of the mode of administration.

Discussion

Work published by other researchers documents an IL-6 response from human PBMC exposed to CPE, but no IL-1, TNF-α, or IFN-γ and no mitogenic response (Krakauer et al., 1997). The lack of a mitogenic response did not concern us greatly because their first evaluation of proliferation was at 48 hours which was, as previously described, long past the point of any observed proliferative effect, and indeed at a time when one would expect the toxic effect of CPE on sensitive cells to be manifested. The lack of an IL-1, TNF-α, or IFN-γ response was of greater interest, however, it is certainly

possible that the researchers missed the production of these cytokines based on the time points chosen for analysis just as they missed the proliferative effect. The levels of IL-1, IL-6, TNF-α, and IFN-γ generated in response to CPE without sensitization were comparable in intensity to those induced by SEB, TSST-1, and LPS in D-gal sensitized mice (Meithke, 1993; Tateda, 1996). Cytokine levels were also faster in induction and more rapid in return to baseline levels than those observed following superantigen challenge. Further studies should be conducted to determine whether CyA-treated mice express TNF-α following CPE challenge. In studies of superantigens, it has been found that inhibiting T-cell function with CyA can completely inhibit systemic TNF-α production, implying that either T-cells produce TNF-α and/or they are involved in the process by which macrophages secrete this cytokine. In the case of CPE challenge, as it appears that macrophages are the principal target with no T-cell involvement, it is expected that CyA will have no effect on the production of TNF-α. As expected, since it is becoming increasingly apparent that CPE is not a superantigen, there was minimal IL-2 generated in response to CPE challenge. TSST-1 and SEB are powerful activators of cells of the macrophage lineage as well as of T-lymphocytes, which upon activation produce a host of inflammatory cytokines. Lipopolysaccharide induced endotoxic shock is primarily a macrophage/monocyte mediated phenomenon, wherein the generalized inflammatory process is attributable to the generation of a cascade of proinflammatory cytokines. Neither TSST-1, SEB, nor LPS are directly toxic to cells, in contrast to CPE which demonstrates a powerful cytotoxic effect in many cell types (Lindsay, 1996). The pathophysiological effects of CPE thus may be due to a combination of direct

cytotoxicity, as well as the generation of a proinflammatory response by the host. The results of this study demonstrate that a macrophage cell line is activated to produce cytokines *in vitro*. This work demonstrates results consistent with an activation of cells of the macrophage lineage *in vivo* as well. Based on the results of the *in vivo* studies, it is apparent, however, that T-cells are not activated to any significant extent following challenge with CPE.

The above outlined studies leave many unanswered questions regarding cytokine production. Additional studies regarding cytokine involvement are suggested by the results of this work some of which were performed and are described in Chapter 5. First, determining the organs of generation of an immune response following CPE intoxication are of interest as is the time-course of the transcriptional response of each affected organ (see Chapter 5). Second, the mediators which serve to down-regulate activity of proinflammatory cytokines should be examined as to transcription, protein expression, time course, and organs of origin. Mediators that regulate cytokine activity are of great interest, as the serum cytokine response observed does not necessarily tell the whole story. Serum cytokines are, in reality, the amount produced in excess of the amount which binds to cellular and soluble receptors. The serum cytokine measurements do not reflect cytokines which are cell associated, and though the manufacturers of the ELISA kits state that soluble receptor bound cytokines are detected quantitatively some researchers have found this claim to be inaccurate. Further studies which would be helpful in the interpretation of the state of immune activation of mice exposed to CPE are suggested. First soluble TNF-α receptor (sTNF-αr) and second IL-2 receptor antagonist (IL-2ra), should be examined. sTNF-αr levels would be informative as TNFα sensitive

cells shed their TNF receptors in response to exposure to TNF. Serum levels of free cytokine are thus lowered relatively rapidly while the plasma levels of its receptor remain elevated long after exposure to a stimulatory agent. sTNF-αr levels therefore can often provide more information about the state of the immune system than cytokine levels. IL-1ra levels would be of interest as IL-1 induces synthesis of this protein, and levels of IL-1ra remain elevated after IL-1 levels have declined. These systems serve a protective function by decreasing the chance that an inflammatory reaction will cause significant damage to the challenged host. The complexity of these interacting systems make interpretation of data solely obtained from measuring serum protein levels difficult. Additional work investigating these systems following CPE intoxication would therefore be of great importance.

CHAPTER 5
ORGAN SPECIFIC TIME COURSE OF CYTOKINE MESSENGER RNA RESPONSE FOLLOWING *CLOSTRIDIUM PERFRINGENS* TYPE A ENTEROTOXIN INTOXICATION

Introduction

The reverse transcriptase polymerase chain reaction (rtPCR) provides a powerful tool to evaluate both the time course and the organ specificity of a transcriptional response. Chapter 4 describes the serum cytokine response to CPE. In order to gain more insight into the mechanisms of response to this toxin, RNA productions for the cytokines previously evaluated (IL-1α, IL-2, IL-6, IFN-γ, and TNF-α) were examined by rtPCR. The organs examined in this manner were the spleen, liver, and small intestine.

Materials and Methods

Reagents

Freeze dried CPE, obtained from Dr. Bruce McClane, was reconstituted, handled, and administered as described in Chapter 3. Reconstitution was performed the day of administration as CPE in solution loses biological activity even when stored at -70^0C. As before protein concentration was determined by the method of Lowry, et al. (1951) with bovine serum albumin as a standard. From this determination the solution was diluted to a final concentration of 1 μg protein/μl diluent with PBS-Tw.

Mice

16.5 ± 1.0 g male Swiss Webster (SW) mice for these studies were obtained through the Department of Animal Resources of the University of Florida from Harlan-Sprague Dawley. Department of Animal Resources personnel ordered, treated, and cared for these animals as described in Chapter 3.

Toxin Administration

Mice were administered CPE essentially as described in Chapter 3. Within each experiment mice were randomly chosen for group assignment. Mice were given 4.5 μg CPE/g body weight in IG administration. This toxin amount is slightly below 0.5 MLD, and was guaranteed to elicit typical pathophysiological symptoms as described in Chapter 3 while not risking the possibility of death.

Necropsy and Organ Harvesting

At the times indicated animals were anesthetized using chloroform and sacrificed by cervical dislocation. Organs harvested included spleen, liver, and distal 5 cm of the small intestine. Organs were immediately placed into 3.0 ml of 4^0C TRIzol reagent (Gibco BRL) in 15 ml polypropylene tubes and homogenized using a Polytron (Brinkmann Instruments) for three bursts of 15 seconds each. This procedure ensured complete homogenization of all organs studied. Homogenates were stored at -70^0C until RNA extraction.

RNA Isolation

Six hundred µl of chloroform was added to 3.0 ml of Trizol:tissue homogenate. This mixture was vortexed to a milky pink color (approximately 15 sec) and allowed to sit at room temperature (25^0C) for 3 min. The sample was then centrifuged in a clinical centrifuge at 2000 x *g* for 60 min at 4^0C. The aqueous phase was transferred to a fresh 15 ml polypropylene tube. 2.0 ml -20^0C isopropyl alcohol was added. The mixture was centrifuged for 20 min in a clinical centrifuge at 4^0C at 2000 x *g*. The supernatant was removed and discarded. The pellet was washed once with 5.0 ml 75% ethanol (made from absolute ethanol plus diethylpyrocarbonate (DEPC) treated water) by gently vortexing and centrifuged as before. The supernatant was removed and discarded and the pellet allowed to air dry for 5 min in a laminar flow hood. RNA was redissolved in 250 µl volumes of DEPC treated water and assayed for RNA concentration and purity by spectrophotometric measurement. RNA concentration was usually 0.7-1.0 µg RNA/µl. If concentration was higher than 1.0 µg RNA/µl the solution was diluted further with DEPC treated water to 1.0 µg RNA/µl. In the rare case that the concentration was lower than 0.7 µg RNA/µl a proportionately larger volume of RNA solution (and correspondingly lower volume of water) was utilized during the reverse transcription reaction.

Reverse Transcription

A 20 µl total reaction volume was used for generating cDNA from the isolated total RNA. All reagents used were obtained from GibcoBRL. Total RNA (1.4-2.0 µg) was used in the reaction. To 2.0 µl RNA solution was added 1.0 µl oligo $(dT)_{12-18}$ (containing 0.5 µg) primer solution and 9.0 µl DEPC H_2O on a -20^0C aluminum block. This mixture was heated to 70^0C for 10 min in a thermocycler (Perkin Elmer) to remove secondary structure from the RNA. The mix was quick chilled on an aluminum block at -20^0C. To this was added a mix containing 4.0 µl 5X first strand buffer, 2.0 µl 0.1 M dithiothreitol (to stabilize the reverse transcriptase), and 1.0 µl of a solution containing 10 mM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP). Contents of each tube were gently mixed and incubated at 42^0C in the thermocycler. To each reaction tube 1µl (200 U) of Superscript II reverse transcriptase was added with gentle pipetting. This mixture was incubated at 42^0C for 50 min followed by an inactivation step of 15 min at 70^0C. The cDNA was stored at -20^0C until use.

PCR Reactions

Sequences for PCR primers were obtained from the lab of Dr. Ammon Peck (Department of Pathology, Immunology, and Lab Medicine, University of Florida) (see Table 5.1 for sequences and product lengths). Primer pairs were analyzed using OLIGO software and the National Library of Medicine database Genebank to ensure specificity and appropriateness. Products for all reactions were of the appropriate length as shown

by agarose gel electrophoresis. PCR primers for β-Actin and the cytokines IL-1α, IL-2, IL-6, IFN-γ, and TNF-α were ordered form GibcoBRL and received in lyophylized, desalted form. They were resuspended in TE8 buffer to a concentration of 50μM. All PCR reactions were conducted using a GeneAmp PCR system 2400 (Perkin Elmer). Before any other PCR reactions were run, the reaction for β-Actin was conducted and the product evaluated by gel electrophoresis to ensure that the RNA isolation and reverse transcription had been conducted successfully. The cycling profile performed was as follows: 35 cycles of [(1) denature 45 sec at 94^0C; (2) anneal 45 sec at 60^0C; (3) extend 2 min at 72^0C] followed by one final 7 min 72^0C extension step. Following evaluation of each cDNA in this manner 5 μl from each of 3 cDNAs from each time period were combined for each organ and PCR reactions were conducted using the same profile as was used for amplifying the segment for β-Actin.

Gel Electrophoresis

Amplified PCR products were separated using 1.5 % agarose gels with 0.7 μg/ml ethidium bromide (see Figs 5.1-5.5 for results). Gels were run with TAE used as the running buffer. To each sample lane was added 10 μl PCR product with 2 μl loading dye (0.25% bromophenol blue, 40% sucrose). The last lane was loaded with 1 μl 100 bp DNA sizing ladder diluted with 8 μl water and 1.5 μl loading dye. Gels were run for 55 min at 95 mV.

Visualization of PCR Products

Visualization of products separated by electrophoresis was performed using a UV transilluminator (Photodyne). Photographic images were produced and the results scanned as high resolution bit-maps.

Results

All reactions produced only one amplification product with the exception of the reaction for TNF-α which frequently produced several extra bands. Although a semiquantitative determination could be made (using two different PCR cycle numbers and visual grading) no additional information would have been obtained and considerably more reagents would have been utilized therefore a simple qualitative analysis was considered adequate.

Results of this work are shown in Figures 5.1-5.5 showing PCR products which are proportional to the relative transcriptional activity for the cytokine genes in question. Figure 5.6 shows a scan of the complete gel of PCR products from the evaluation of IL-1α mRNA from liver.

IL-1 mRNA was generated primarily in the spleen with lesser amounts being produced by the liver and a transient transcription induced in the small intestine (see Fig 5.1).

As expected based on the prior work virtually no IL-2 mRNA was induced although there was a slight response seen from the spleen at times 60 and 120 min that did not reproduce well in the photographs of the gels (Fig 5.2).

The IL-6 transcriptional response was most pronounced in the liver but this response had ended long before the serum protein levels declined (see Fig 5.3).

Maximum serum levels of this cytokine were reached within 60 min and remained at maximum levels until the 8 h time point. Maximum mRNA levels were reached in the same time and then rapidly declined.

The most vigorous IFN-γ transcription was seen in the small bowel with lesser amounts generated in the spleen and liver (Fig 5.4). Maximum serum protein levels peaked very rapidly (by the 60 min time point) and then declined very rapidly. The transcriptional response was induced rapidly reaching maximum intensity within 30 min however the transcriptional levels remained elevated throughout the time course examined remaining at maximum intensity even at the 8 h time point.

Detectable TNF-α serum protein levels were relatively low throughout the course of the experiment with a slight amount seen which slowly declined throughout the experiment. Despite this paucity of detectable cytokine there was a vigorous transcriptional response for this cytokine (Fig 5.5), primarily in the liver but also in the spleen and bowel.

Discussion

Despite the fact that the serum levels of IL-1α had returned to undetectable levels by 8 hr after IG administration of toxin there was still a vigorous IL-1α transcriptional

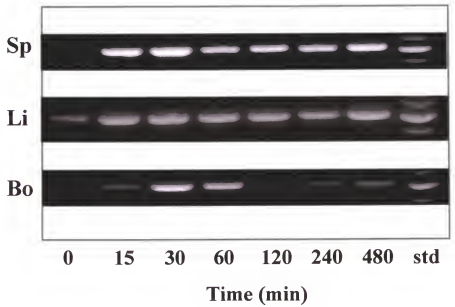
response in the spleen and the liver. The bowel demonstrated a transient response which was evident at only the 30 and 60 min time points. It is possible that there is a post-transcriptional regulation of this protein through the production of IL-1 receptor antagonist (sIL-1ra) which binds to the IL-1 receptor, attenuating the inflammatory response.

Table 5.1. Cytokine primers used for PCR

Cytokine		Primer Sequence	Product size (bp)
IL-1α	3' Primer	5' CCTTCAGCAACACGGGCTGGTC 3'	625
	5' Primer	5' ATGGCCAAAGTTCCTGACTTGTTT 3'	
IL-2	3' Primer	5' GGCTTGTTGAGATGATGCTTTGACA 3'	502
	5' Primer	5' ATGTACAGCATGCAGCTCGCATC 3'	
IL-6	3' Primer	5' CACTAGGTTTGCCGAGTAGATCTC 3'	638
	5' Primer	5' ATGAAGTTCCTCTCTGCAAGAGACT 3'	
IFN-γ	3' Primer	5' CGACTCCTTTTCCGCTTCCTGAG 3'	460
	5' Primer	5' TGAACGCTACACACACTGCATCTTGG 3'	
TNF-α	3' Primer	5' CCAAAGTAGACCTGCCCCGGACTC 3'	692
	5' Primer	5' ATGAGCACAGAAAGCATGATCCGC 3'	
β-ACTIN	3' Primer	5' CTCTTTGATGTCACGCACGATTTC 3'	540
	5' Primer	5' GTGGGCCGCTCTAGGCACCAA 3'	

Further experiments should examine both the serum protein levels of this cytokine receptor and the transcriptional response for the mRNA for sIL-1ra.

IL-2 mRNA was generated in only trace amounts by the spleen and mirrors the vanishingly small amounts of IL-2 detected in serum of CPE challenged mice. This



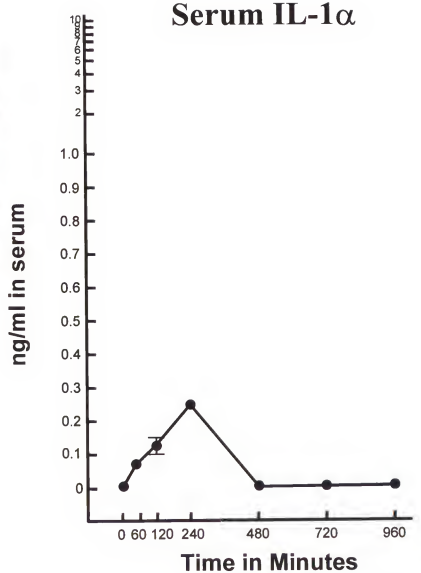


Figure 5.1 IL-1α mRNA Evaluation

rtPCR evaluation of mRNA for IL-1α following IG administration of 0.5 MLD of CPE showing time course and organ specificity of transcription (above). Serum levels of IL- 1α are also shown (right).
Sp =Spleen Li=Liver Bo=Small Bowel
MLD=Mouse Lethal Dose
CPE=*Clostridium perfringens* type A enterotoxin

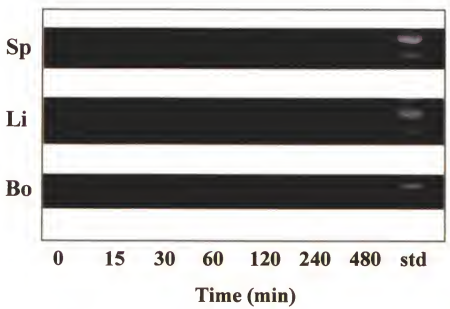
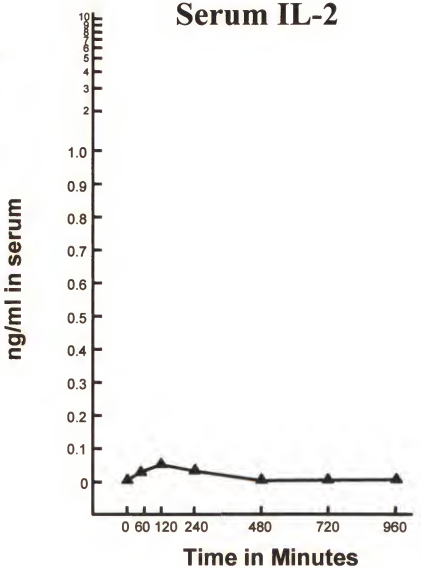
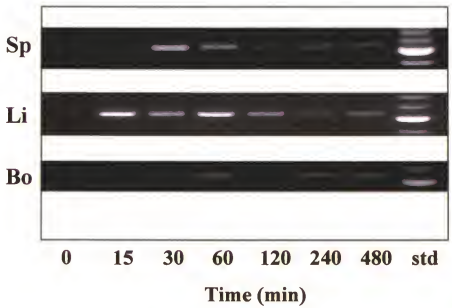


Figure 5.2 IL-2 mRNA Evaluation

rtPCR evaluation of mRNA for IL-2 following IG administration of 0.5 MLD of CPE showing time course and organ specificity of transcription (above). Serum levels of IL- 2 are also shown (right).
Sp =Spleen Li=Liver Bo=Small Bowel
MLD=Mouse Lethal Dose
CPE=*Clostridium perfringens* type A enterotoxin





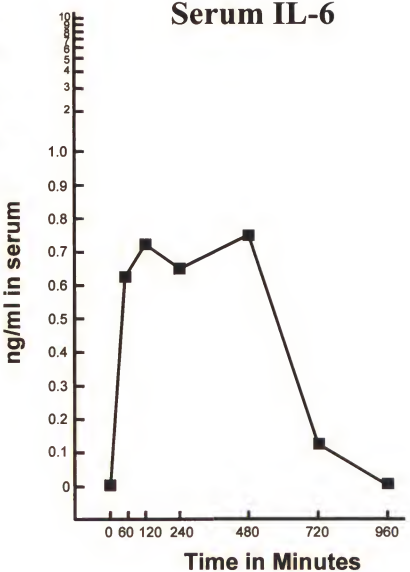
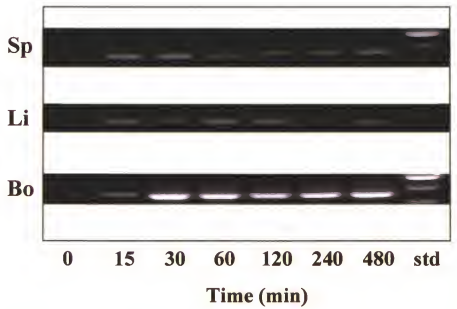


Figure 5.3 IL-6 mRNA Evaluation

rtPCR evaluation of mRNA for IL-6 following IG administration of 0.5 MLD of CPE showing time course and organ specificity of transcription (above). Serum levels of IL-6 are also shown (right).
Sp =Spleen Li=Liver Bo=Small Bowel
MLD=Mouse Lethal Dose
CPE=*Clostridium perfringens* type A enterotoxin



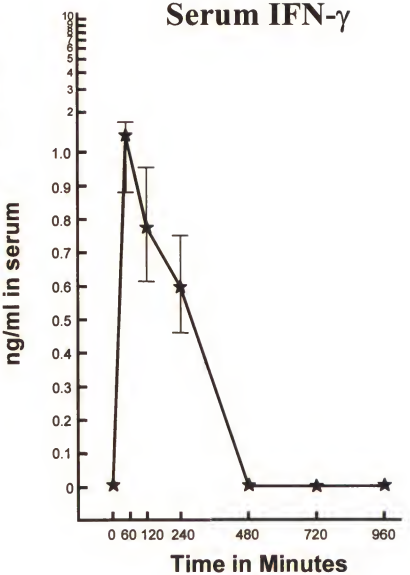
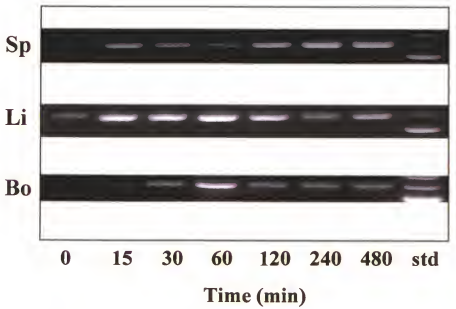


Figure 5.4 IFN-γ mRNA Evaluation

rtPCR evaluation of mRNA for IFN-γ following IG administration of 0.5 MLD of CPE showing time course and organ specificity of transcription (above). Serum levels of IFN-γ are also shown (right).
Sp =Spleen Li=Liver Bo=Small Bowel
MLD=Mouse Lethal Dose
CPE=*Clostridium perfringens* type A enterotoxin



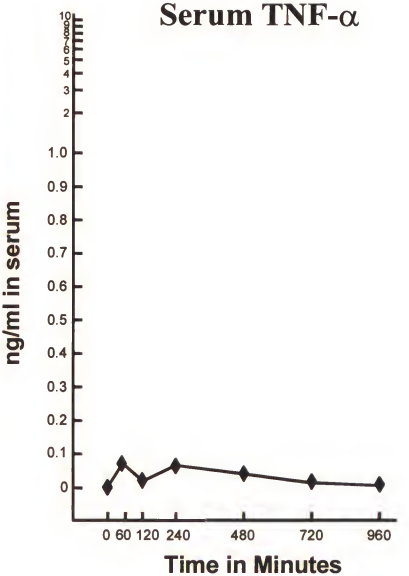


Figure 5.5 TNF-α mRNA Evaluation

rtPCR evaluation of mRNA for TNF-α following IG administration of 0.5 MLD of CPE showing time course and organ specificity of transcription (above). Serum levels of TNF-α are also shown (right).
Sp =Spleen Li=Liver Bo=Small Bowel
MLD=Mouse Lethal Dose
CPE=*Clostridium perfringens* type A enterotoxin

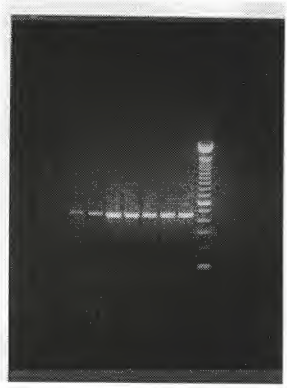


Figure 5.6 RT-PCR evaluation of liver transcription for IL-1α.

result confirms that T-cells are not activated to any great extent in the murine model of CPE intoxication.

IL-6 transcription, unlike that for IL-1α, had decreased long before any decrease in IL-6 serum protein was evident with a major decline between the 60 and 120 min time points. In contrast, serum protein levels of this cytokine did not begin to decline from their maximal amounts until after the 8 h time point. This indicates that likely the regulation of this cytokine following CPE intoxication is at the transcriptional level.

Transcription of IFN-γ was most pronounced in the bowel with lesser amounts being produced by the spleen and liver. As with IL-1α mRNA for IFN-γ remained elevated after serum protein levels had returned to baseline levels indicating a post-transcriptional regulation of the activity of this cytokine.

TNF-α presents the most confusing picture of all with spleen, liver and bowel all being transcriptionally active for this cytokine. The liver is most transcriptionally active with regard to TNF-α with lesser amounts but the same time course evident for the bowel. In contrast splenic production of TNF-α mRNA is weak initially but increases throughout the timecourse of the experiment peaking at 4 h and remaining at that level at the 8 h time point. Compared with the relatively small amount of this cytokine seen in serum following CPE challenge the vigorous transcriptional response is of great interest. It is quite possible that shed TNF-α receptors are masking the true levels of serum protein. Because of this result it is important to evaluate the serum levels of soluble receptor for this cytokine in future work using the same experimental design as that utilized in Chapter 4, which may provide a more complete understanding of the kinetics of TNF-α expression in CPE intoxication.

These findings that the gut demonstrated cytokine gene transcription are in contrast to experiments modeling endotoxicosis. Work conducted to evaluate the gut as a source of cytokines during a porcine model of LPS exposure indicated that the gut was not a source of cytokines (Bathe, 1996). The work outlined here evaluated transcriptional activity which may not be a true indicator of protein production, while the endotoxemia model system evaluated actual protein production which does make direct comparisons

between these experiments impossible. Should later work provide evidence that cytokines are actually produced by the gut during CPE intoxication, then further studies investigating the difference in mechanisms between these two states would be warranted.

CHAPTER 6
FECAL BILE ACIDS, NEOPTERIN, *CLOSTRIDIUM PERFRINGENS* AND THE SUDDEN INFANT DEATH SYNDROME

Introduction

In humans, illness caused by the *Clostridium perfringens* enterotoxin (CPE) manifests itself in two ways. First and most common is the form caused by ingestion of large numbers ($>10^7$) of viable vegetative cells. These cells, under the stressful environment encountered in the gastrointestinal tract, are induced to sporulate with the associated production of enterotoxin. Symptoms begin 8-24 hours after eating contaminated food. The enterotoxin acts on the epithelium of the small intestine to induce a prolific non-bloody secretory diarrhea with desquamation of enterocytes primarily in the distal ileum. This illness resolves spontaneously within 12-24 hours of the onset of symptoms. The second and more aggressive illness is the Infectious Diarrhea Syndrome which occurs primarily in institutional settings (nursing homes, long-term care facilities).

Increased frequency and levels of *C. perfringens* spores and CPE have been demonstrated to occur in the feces of victims of the Sudden Infant Death Syndrome (SIDS) and it has been proposed that the sporulation associated CPE could be at the least a contributing factor (if not the final trigger) in some cases of SIDS. Bile acids are known to induce sporulation of vegetative cells of *C. perfringens* (Heredia et al., 1991).

Total bile acid levels and individual bile acids were examined in SIDS cases to determine if increased levels were associated with SIDS.

In humans activation of the cellular branch of the immune system is accompanied by the release of neopterin (tetrahydrobiopterin). This effect is mediated through IFN-γ. An increased neopterin level indicates endogenous interferon has been produced and therefore neopterin levels can be used as a measure of the activated state of the immune system. Neopterin has been shown to be involved in a variety of processes including the oxidative cleavage of ether lipids and the formation of nitric oxide radical from arginine (Fuchs et al., 1992). *In vitro* studies utilizing a macrophage cell line have indicated that CPE induces a vigorous mitogenic and cytokine response. *In vivo* studies of cytokine production, as discussed in previous chapters, indicate that this response happens in macrophages of animals as well. As such, if CPE were involved in SIDS, one would expect to see increased levels of neopterin in SIDS victims. Neopterin levels remain elevated long after cytokine levels have decreased and are stable in urine post-mortem (Ambach et al., 1991). Neopterin levels are thus an ideal marker to study whether the cellular branch of the immune system has been activated in SIDS. While not indicative of a causal relationship between CPE and SIDS, elevated neopterin levels in SIDS victims would be suggestive of a link between the elevated levels of fecal *C. perfringens* spores and CPE found in SIDS victims and the immunological state of these infants. Conversely, a finding of no increased levels of neopterin in SIDS victims would suggest that the elevated levels of *C. perfringens* have no importance in the phenomenon of SIDS and are merely associated without being causal in nature.

Materials and Methods

Fecal and Urine Samples

Feces and urine were obtained from SIDS victims and from live controls. Feces from 18 SIDS victims (15 *C. perfringens* positive and 3 *C. perfringens* negative) and 10 control infants (5 *C. perfringens* positive and 5 *C. perfringens* negative) were examined for bile acid content. The sex of the infant was not considered as a factor. Samples were stored at -20 °C until analysis. Samples for analysis were coded and analyzed in a blind fashion without knowledge as to which group they belonged. Fecal samples from live, healthy babies of matching age and environment who were born at term, were gaining weight normally, and were not taking medication or antibiotics were also examined. These infants were considered likely to give an accurate picture of the normal bile acid levels in feces. This matching methodology has been used previously in other SIDS studies as a means of comparison (Telford et al., 1989; Bettelheim et al., 1990; Lindsay et al., 1993).

Urine from 7 SIDS victims and two victims of traumatic death were obtained. Samples were collected by bladder puncture from the dead infants. Because neopterin is light sensitive samples were collected into either foil-covered plastic vials or into opaque brown plastic vials. Samples were stored at -70 °C until analysis.

Microbiology

The methodology of Lindsay et al. (1993) was used for microbiological analysis. *C. perfringens* total viable counts (TVC) of vegetative cells were determined by pour plating of serially diluted fecal samples in duplicate on tryptone-sulfite-neomycin (TSN)

agar (BBL, Cockeysville, Md.) and incubating anaerobically at 45 °C for 48 hours. TVC of spores were determined by heating a sample at 75 °C for 20 min before plating. TSN is a differential and selective media for *C. perfringens* with colonies of this organism having a characteristically black color. In this study a TVC of $>10^5$ for vegetative cells and $>10^3$ for spores was considered presumptive positive. Confirmation of *C. perfringens* spores was given with a positive ELISA result for CPE with levels of >1ug toxin/g feces (Lindsay et al., 1993).

Enterotoxin Quantification

ELISA for CPE was performed. Briefly, fecal samples were diluted 1:10 with 0.1M phosphate buffer pH 6.7 containing 0.85% NaCl, incubated at 25 °C for 20 min, then centrifuged at 20,000 *g* for 15 min at 4°C. The supernatant was collected and analyzed in triplicate in 96 well Immulon-2 plates. After sample incubation and washing, attachment of the primary antibody (rabbit anti-CPE) and secondary (goat anti-rabbit alkaline phosphatase conjugate) antibodies and washing the antigen-antibody complex was detected by the alkaline phosphatase reaction measured after 4 h at 405nm. This procedure was found to have a lower limit of 50 pg (Lindsay et al., 1993).

Bile Acid Analysis

Bile acid extraction and analysis was performed using a combination of published methods (Locket et al., 1989; Roda et al., 1992; Setchell et al., 1983). To 100 mg feces, 400 ul 0.01 N HCl was added in a 15 ml glass screw cap test tube and vortexed for five

min. 1.6 ml ethanol was added to the sample and vortexed briefly followed by sonication for 15 min. The sample was then capped tightly and refluxed at 100 °C for 15 min. The sample was cooled and centrifuged at 5000x*g* for 10 min. The supernatant was removed and retained. The pellet was resuspended by vortexing and then sonicating for 15 min in 2.0 ml 80% aqueous ethanol, capped, and refluxed for 15 min. The sample was again cooled and centrifuged as before and the supernatant removed and retained. The pellet was then resuspended in 50% chloroform/50% methanol by vortexing briefly and sonicating for 10 min. The solution was refluxed as before for 15 min and centrifuged as before. The supernatant was removed and retained. The pellet was scraped onto filter paper and washed with a small volume of 50% chloroform/50% methanol. All extracts were combined and taken to dryness under nitrogen at 42 °C.

The dried extract was resuspended in 1.0 ml 0.01 N HCl and sonicated for 10 min. Chloroform (2.0 ml) was added to extract unconjugated bile acids. The chloroform was removed and the sample dried under nitrogen at 42 °C. The aqueous phase was passed through an activated Bond-Elut cartridge of reversed phase octadecylsilane (Analtichem International, Harbor City, Ca), washed with 5 ml 0.1 N HCl, and then eluted with 2.0 ml methanol to remove conjugated bile acids. The chloroform and methanol fractions were dried together under nitrogen at 42 °C, and resuspended in 0.6 ml volume of the initial mobile phase. HPLC analysis was performed using a Perkin Elmer Series III Chromatograph equipped with a Nova-pac 3.9 x 300 mm C-18, 4 μm particle size column. The column was kept at a constant 37 °C using a water jacket. The detector was an evaporative light scattering mass detector (ELSD II) (Varex Co. Burtonville, Md). A constant flow rate of 0.9 ml/min was used. The initial mobile phase was 65% (v/v)

aqueous methanol containing 15 mM ammonium acetate adjusted to a pH of 5.4 ± 0.1 with acetic acid. Fifteen min after injection of 200 µl of the resuspended extract, a 40 min convex gradient to 85% methanol with 15 mM ammonium acetate (pH 5.4 ± 0.1) was run. This methodology gave good separation of each bile acid examined. The peaks for taurocholic acid (TCA), glycocholic acid (GCA), cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDA), and lithocholic acid (LCA) were quantitated. The detection limit was < 10 nM for each bile acid, which is approximately 3 µg/100 mg feces. Two samples for each case were examined, and each sample was run in duplicate. Samples for each bile acid treated in this manner gave > 95% recovery. A standard curve was generated before each series of samples was run. Standards were obtained from Sigma Statistical analysis was performed using the Kruskal-Wallis test for nonparametric data.

Neopterin analysis

A neopterin standard was made by dissolving 10 mg crystalline neopterin in 800 ml distilled, deionized, milli-Q filtered water with gentle heating and constant stirring. The sample was cooled slowly to 25 °C, diluted to 1000 ml, aliquoted into 100 ml opaque containers, and stored at –70 °C until used.

Neopterin and creatinine concentrations were determined in one run in series by high-pressure liquid chromatography. Neopterin concentrations were normalized to creatinine concentrations to account for variations in urine density. All urine samples were diluted 100-fold in potassium phosphate buffer (15 mmol/L, pH 6.4) for analysis and 100 µl of this solution was injected for analysis. A Perkin-Elmer HPLC system with

a Supelcosil reversed phase LC-18 column (25 cm x 4.6 mm) with 5 μm bead size was used. Detectors included a Perkin-Elmer diode array detector for measurement of neopterin and a Dionex UV absorption detector for measurement of creatinine. Neopterin was detected by its native fluorescence with 353 nm excitation and 438 nm emmision. Assays were run at 25 °C using a 0.8 ml/min flow rate in an isocratic buffer of the same composition as the diluent used for diluting the urine samples (potassium phosphate buffer 15 mmol/L, pH 6.4).

Results

Examples of typical chromatograms for samples and standards are shown in Figure 5.1. Results for total fecal bile acids for the four categories (SIDS/CP+, SIDS/CP, Control/CP+, Control/CP-) are shown in Figure 5.2. The n value for SIDS CP/- is low which is simply a function of the small population of such SIDS victims. The mean values (μg/g feces) for the four categories are SIDS/CP+:310, SIDS/CP-:127, Control/CP+:, Control/CP-:304. These levels are similar to results obtained in a longitudinal study of total fecal bile acids in infants of various ages (Hammons et al., 1988). Comparisons between 1. SIDS CP+/- vs Control CP+/- data, and 2. SIDS CP+ plus Control/CP+ vs SIDS CP- plus Control/CP- showed no significant differences between the rank sums of total bile acids in either case. Analysis of individual bile acids using these comparisons and the same statistical procedures also indicated no significant differences. Taurine conjugates and glycine conjugates were rarely observed in detectable amounts. There was also no difference between total primary and total secondary bile acid excretion in SIDS vs controls, and CP+ vs CP- cases.

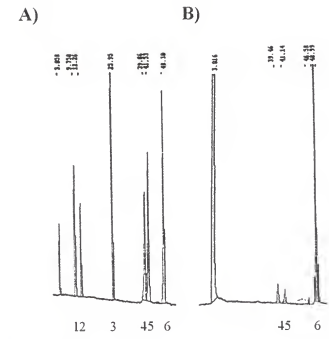


Figure 6.1 Chromatograms for (A) Standards (B) Representative fecal sample.

1. Taurocholate 2. Glycocholate 3. Cholate
4. Chenodeoxycholate 5. Deoxycholate 6. Lithocholate

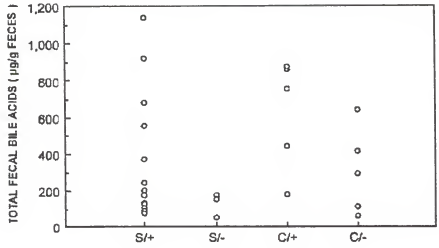


Figure 6.2 Total fecal bile acid levels.
S/+ (SIDS/CP+), S/- (SIDS/CP-), C/+ (Control/CP+), C/- (Control/CP-)

Results of the neopterin study were inconclusive because of the small sample size, but suggestive. In seven studied cases of SIDS the urinary neopterin level (in μmol neopterin/mol creatinine) was 2208 ± 574 (mean ± S.D.). Two control infants demonstrated neopterin levels of 320 and 581.

Discussion

Despite considerable evidence of a role for *C. perfringens* in SIDS, the means whereby the organism sporulates and produces toxins in the gut is not completely understood. This is extremely important in understanding the etiology of SIDS because cause and effect relationships can be especially difficult to elucidate in these cases. Even a cursory review of the SIDS literature indicates that there are many detectable abnormalities in the SIDS population which makes ascertaining a correlation quite different from establishing causality. To cause enteric pathology, *C. perfringens* must sporulate and interestingly certain bile acids, including taurocholate, are known to stimulate sporulation (Floch et al., 1972; Hickey and Johnson, 1981; Phillips, 1986; Ushijima, 1986; Labbe, 1989). According to Gaull (1983) taurine is a conditionally essential amino acid which is essential during the first few months of life. The formation of cholate conjugates with taurine and glycine in the infant was suggested to be proportional to body weight and normally fully developed after several months. There is a sparing of infants from SIDS during the first month of life. It is interesting to speculate that this may be due to the lower levels of certain bile acids during this period being insufficient to trigger sporulation of *C. perfringens* and production of enterotoxin.

This study sought to determine whether fecal bile acid (and in particular taurine and glycine conjugate levels) concentrations could be responsible for the high levels of *C. perfringens* spores and CPE seen in many SIDS cases. The results obtained do not support the hypothesis that there is any connection. In fact significant taurine and glycine conjugate levels were not observed in any sample, SIDS or control. The vast majority of bile acids were unconjugated (possibly due to deconjugation of the bile acids following collection), with CA, CDA, and LCA predominating. This result is consistent with an earlier study in humans, where bile acids were artificially raised without any apparent difference in anaerobic flora (Williams et al., 1975). *In vitro* studies, however, have demonstrated a general inhibitory effect of elevated bile acid levels on growth, yet a stimulatory effect on sporulation and enterotoxin synthesis by *C. perfringens*. Why this effect should not be observed *in vivo* is not clear. The in vitro studies indicated that each bile acid (sodium cholate, sodium taurocholate, sodium glycochenodeoxycholate, and sodium chenodeoxycholate) inhibited growth to a different degree, while a mixture completely inhibited growth (Heredia et al., 1991). Other studies have shown that high concentrations of sodium taurocholate stimulated sporulation (Ushijima, 1986), however a similar effect in the above mentioned study was observed only at low but not high concentrations (Heredia et al., 1991). Another study, though, found no stimulatory effect of this salt on sporulation (Hickey and Johnson, 1981). Differences in the results of these studies have been suggested to perhaps be due to strains used and media employed (Heredia et al., 1991). Interestingly, sodium taurocholate is often incorporated into media for the selective recovery of clostridia (Wilson et al., 1982), while a sporulation media for *C. perfringens* containing ox bile has been reported (Phillips, 1982).

Excretion of *C. perfringens* spores and CPE within feces is an excellent indicator of both food poisoning and SIDS (Skjelkvale and Uemura, 1977; Lindsay et al., 1993), however fecal bile acid levels appear not to be correlated with either CP, CPE, or SIDS. Perhaps an examination of ileal bile acids would be more informative. Depressed levels of bile acids could prove to be a risk factor for *C. perfringens* colonization of the gut. Alternately, transiently increased levels of ileal bile acids may be an inducer of sporulation as was previously suggested. It thus appears that fecal bile acids *per se* cannot be associated with increased levels of *C. perfringens* spores and CPE.

The neopterin portion of this study was hampered by several complicating factors. First, the proportion of SIDS victims with urine in their bladders at the time of death was less than one half. Second, within the last ten years, likely as a result of the campaign to place babies on their backs or sides to sleep, the SIDS incidence has fallen dramatically from a rate of almost 2 per 1000 live births in 1989 (Willinger, 1989) to approximatly 1 per 1000 live births today (Scott et al., 1998). Third, control infants who die of a non-infectious cause are extremely rare making obtaining proper controls a difficult task.

In this rather limited study neopterin levels far above those considered normal were seen in infants who had died of SIDS. These levels provide evidence that there is indeed an activation of the cell-mediated arm of the immune system, in particular cells of the monocyte/macrophage lineage, in SIDS. These results are consistent with the finding that infants succumbing to SIDS had elevated levels of IL-6 in their cerebrospinal fluid providing evidence of an activated immune system (Vege et al., 1995). A larger study of this phenomenon would thus appear warranted.

CHAPTER 7
CONCLUSIONS

In this work immune response to the *Clostridium perfringens* type A enterotoxin (CPE) was examined using the mouse model. Initially it was believed that CPE was a superantigen and would thus likely mediate its effects at least in part through T-cell activity. Though this was found to not be true, work done *in vitro* indicated that it was a potent activator of macrophages (Wallace et al., 1999).

The cytokines levels examined in this work demonstrated that there was a strong pro-inflammatory response to the *C. perfringens* type A enterotoxin. The spectrum of cytokines induced mirrors closely the cytokine profile produced following LPS exposure. The cytokine protein response ends relatively rapidly when one compares this response with that obtained following challenge with either LPS or with a staphylococcal suparantigneic enterotoxin. This work utilized mice that were not sensitized with D-gal as are the mice in experiments involving the other toxins. It is certainly possible that the perturbation of the immune system by D-gal lengthens the response to bacterial toxins. Other researchers have used these sensitized mouse models since they more closely mimic the immune response observed in humans, as to concentration required to generate a pathophysiological response or type of response obtained (Miethke et al., 1993). To date no work has been performed in non-stimulated mice as to cytokine time course following exposure to either LPS or the staphylococcal enterotoxins. It should be considered that CPE interferes with macromolecular synthesis and viability in sensitive

cells including those of the monocyte/macrophage lineage (Wallace et al., 1999). As it appears from the work outlined here that the CPE induced proinflammatory response is primarily a result of these cells then it also is possible that synthesis of these cytokines is a self-limiting phenomenon.

The finding that the liver and bowel are transcriptionally active for cytokines during CPE intoxication is in agreement with previous results which found high binding to tissues from these organs (Keller, 1997).

Serum cytokines decline before CPE is eliminated from the body in the mouse model (Keller, 1997). Free CPE is still detectable in thymus, bowel, and heart 72 hours after IG administration of 0.5 MLD, the amount used in these studies. The inflammatory response is thus attenuated through as yet undetermined means. Three possibilities, not mutually exclusive, are suggested to explain this phenomenon. 1) Cytokine transcription is downregulated. This appears to be the case with IL-6 in which serum proteins remain elevated long after transcription of this cytokine has ceased. 2) Free cytokines are bound by soluble receptors. This appears likely in the case of IL-1α as transcription of this cytokine remains high through the 8 h time point despite the fact that detectable serum protein levels begin to decline after the 4 h time point and have reached baseline levels by the 8 h determination. 3) Third, sensitive cells, including a macrophage cell line, show decreased viability following the initial mitogenic effect observed following CPE exposure. This lethal effect is almost assuredly the result of the pore-forming activity and subsequent inhibition of macromolecular synthesis demonstrated by this toxin. It thus appears likely that the inflammatory response generated by CPE is a self-limiting

phenomenon. There appear to be controls built into the cytokine expression system at both the transcriptional level as well as the protein expression level either through translational controls or through the synthesis or release of mediators which bind free cytokines. Also cells generating the response are killed after an initial period of heightened activity and thus would not contribute to further inflammatory activity (Wallace et al., 1999).

In conclusion, CPE has been found to generate an immune response which is of a type found to be similar to that observed following exposure to LPS and the staphylococcal enterotoxins. This response appears, when one examines transcription of mRNA for cytokines, to be the result of multiple organs including spleen, liver, and bowel. In the food-borne CPE intoxication this cytokine response could play a role in the accompanying nausea. In the infectious diarrhea syndrome there is a more protracted exposure to CPE. The greater tissue damage evident during this illness could in fact be due to resultant inflammatory processes mediated by cytokines. Clinical research investigating this possibility should be undertaken as should animal model studies. Additionally, if CPE is involved in the sudden infant death syndrome then this work could be of great value in proving this hypothesis. A role for bile acids in SIDS was not supported by this research. The cytokines examined and their profiles suggest that further research should be conducted on the phenomenon of SIDS examining products related to CPE exposure which persist after the initial rapid response. A large enough series of urine samples should be evaluated for neopterin levels to make an evaluation of

whether the heightened levels seen in our small preliminary study are present in those amounts in a larger and statistically significant group of SIDS victims.

REFERENCES

Ahtonen P, Lehtonen OP, Kero P, Eerola E, Hartiala K (1994) *Clostridium perfringens* in stool, intrapartum antibiotics and gastrointestinal signs in a neonatal intensive care unit. Acta Paediatr 83:389-390.

Ambach E, Tributsch W, Fuchs D, Reibnegger G, Henn R, Wachter H (1991) Postmortem evaluation of serum and urine neopterin concentrations J Forensic Sci 36:1089-1093.

Ando Y, Tsuzuki T, Sunagawa H, Oka S (1985) Heat resistance, spore germination, and enterotoxigenicity of *Clostridium perfringens*. Microbiol Immunol 29:317-326.

Baron S, Tyring SK, Fleischmann WR, Coppenhaver DH, Neisel DW, Klimpel GR, Stanton J, Hughes TK (1991) The interferons: mechanisms of action and clinical applications. JAMA 266:1375-1383.

Barton BE, Jackson JV (1993) Protective role of interleukin 6 in the lipopolysaccharide-galactosamine septic shock model. Infect Immun 61:1496-1499.

Bathe OF, Rudston-Brown B, Chow AWC, Phang PT (1996) Gut is not a source of cytokines in a porcine model of endotoxicosis Surgery 120:522-533.

Beagley KW, Elson CO (1992) Cells and cytokines in mucosal immunity and inflammation. Gastroenterol Clin N Am 12:347-366.

Beckwith JB (1988) Intrathoracic petechial hemorrhages: a clue to the mechanism of death in sudden infant death syndrome? Ann NY Acad Sci 533:37-47.

Bejarano MT, Masucci MG, Klein E (1986) Differential effect of Cyclosporin-A on the mixed-lymphocyte culture-induced proliferative and cytotoxic responses of T-lymphocytes with different cell densities. Cell Immunol 103:409-416.

Belardelli F (1995) Role of interferons and other cytokines in the regulation of the immune response. APMIS 103:161-179.

Bemelmans MHA, van Tits LJH, Buurman WA (1996) Tumor necrosis factor: function release and clearence. Crit Rev Immunol 16:1-11.

Bette M, Shafer MKH, van Rooijen N, Weihe E, Fleischer B (1993) Distribution and kinetics of superantigen-induced cytokine gene expression in mouse spleen. J Exp Med 178:1531-1540.

Bettelheim LA, Goldwater PN, Dwyer BW, Bourne AJ, Smith DL (1990) Toxigenic *Esherichia coli* associated with SIDS. Scand J Infect Dis 22:467-476.

Bhakdi S, Muhly M, Korom S, Schmidt G (1990) Effects of *Escherichia coli* hemolysin of human monocytes. J Clin Invest 85:1746-1753.

Bowness P, Moss PAH, Tranter H, Bell JI, McMichael AJ (1992) *Clostridium perfringens* enterotoxin is a superantigen reactive with human T cell receptors Vβ6.9 and Vβ22. J Exp Med 176:893-896.

Brynskov J, Nielesen OH, Ahnfelt-Ronne I, Bendtzen K (1992) Cytokines in inflammatory bowel disease. Scand J Gastroenterol 27:897-906.

Bunjes D, Hardt C, Rollinghoff M, Wagner H (1981) Cyclosporin A mediates immunosuppression of primary cytotoxic T cell responses by impairing the release of interleukin 1 and interleukin 2. Eur J Immunol 11:657-661.

Byard RW (1991) Possible mechanisms responsible of SIDS. J Pediatr Child Health 27:147-157.

Canard B, Saint-Joanis B, Cole ST (1992) Genomic diversity and organization of virulence genes in the pathogenic anaerobe *Clostridium perfringens*. Molc Microbiol 6:1421-1429.

Cavaillon JM (1994) Cytokines and macrophages. Biomed Pharmacother 48:445-453.

Cerami A (1992) Inflammatory cytokines. Clin Immunol Immunopath 62:S3-S10.

Cohen MC, Cohen S (1996) Cytokine Function: a study in biologic diversity. Am J Clin Pathol 105:589-598.

Collee JG (1974) *Clostridium perfringens* (*C. welchii)* in the human gastrointestinal tract. Soc Appl Bacteriol Symp Ser 3:205-219.

Collie RE, McClane BA (1998) Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal disease. J Clin Microbiol 36:30-36.

Cornillot E, Saint-Joanis B, Daube G, Granum PE, Canard B, Cole ST (1995) The enterotoxin gene (cpe) of *Clostridium perfringens* can be chromosomal or plasmid-borne. Molecular Microbiol 15:639-647.

Cox G, Gauldie J (1997) Interleukin-6. In: Remick DG, Frieland JS (eds) Cytokines in health and disease. New York.: Marcel Dekker, Inc pp 81-99.

Czeczulin JR, Hanna PC, McClane BA (1993) Cloning, nucleotide sequencing, and expression of the *Clostridium perfringens* enterotoxin gene in *Eschericia coli*. Infect Immun 61:3429-3439.

Dinarello CA (1992) Role of Interleukin-1 in infectious diseases. Immun Rev 127:119-146.

Dugas B, Mossalayi MD, Damais C, Kolb J-P (1995) Nitric oxide production by human monocytes: evidence for a role of CD23. Immunol Today 16:574-579.

Duncan CL, Strong DH (1968) Improved medium for sporulation of Clostridium perfringens. Appl Microbiol 16:82-89.

Duncan CL, Strong DH (1969) Ileal loop fluid accumulation and production of diarrhea in rabbits by cell-free products of *Clostridium perfringens*. J Bacteriol 100:86-94.

Eigler A, Sinha B, Hartmann G, Endres S (1997) Taming TNF: strategies to restrain this proinflammatory cytokine. Trends Immunol 18:488-492.

Fantone JC (1997) Cytokines and neutrophiles: Neutrophile derived cytokines and the inflammatory response. In: Remick DG, Frieland JS (eds) Cytokines in health and disease. New York.: Marcel Dekker, Inc pp 373-380.

Farner NL, Hank JA, Sondel PM (1997) Interleukin-2: molecular and clinical aspects. In: Remick DG, Frieland JS (eds) Cytokines in health and disease. New York.: Marcel Dekker, Inc pp 29-40.

Ferrini S Moretta A, Biassoni R, Nicolin A, Moretta L (1986) Cyclosporin-A inhibits IL-2 production by all human T-cell clones having this function, independent of the T4/T8 phenotype or the coexpression of cytolytic activity. Clin Immunol Immunopathol 38:79-84.

Fisher MM, Yousef IM (1973) Sex differences in the bile acid composition of human bile: studies in patients with and without gallstones. Can Med Assoc J 109:190-193.

Flegel WA, Muller F, Daubener W, Fischer HG, Hadding U, Northoff H (1991) Cytokine response by human monocytes to *Clostridium difficile* toxin A and toxin B. Infect Immun 59:3659-3666.

Floch MH, Binder HJ, Filburn BF, Gershengoren W (1972) The effect of bile acids on intestinal microflora. Amer J Clin Nut 25:1418-1426.

Fong Y, Tracey KJ, Moldawer LL, Hesse DG, Manogue KB, Kenney JS, Lee AT, Kuo GC, Allison AC, Lowry SF, Cerami A (1989) Antibodies to cachectin/tumor necrosis factor reduce interleukin 1β and interleukin 6 appearance during lethal bacteremia. J Exp Med 170:1627-1633.

Freudenberg MA, Keppler D, Galanos C (1986) Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. Infect Immun 51:891-895.

Frieben WR, Duncan CL (1973) Homology between enterotoxin and spore structural protein *Clostridium perfringens*. Eur J Biochem 39:393-401.

Fuchs D, Weiss G, Reibnegger G, Wachter H (1992) The role of neopterin as a monitor of cellular immune activation in transplantation, inflammatory, infectious, and malignant diseases. Crit Rev Clin Lab Sci 29:307-341.

Galanos C, Freudenberg MA, Reutter W (1979) Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc Natl Acad Sci (USA) 76:5939-5943.

Galanos C, Freudenberg MA (1993) Mechanisms of endotoxin shock and endotoxin hypersensativity. Immunobiol 187:346-356.

Garcia-Alvarado JS, Labbe RG, Rodriguez MA (1992) Sporulation and enterotoxin production by *Clostridium perfringens* Type A at 37 and 43°C. App Environ Microbiol 58:1411-1414.

Gaull GE (1983) Taurine in human milk: growth modulator or conditionally essential amino acid? J Pediatr Gastroenterol 2:S266-S271.

George WL, Finegold SM (1988) Clostridia in the human gastrointestinal flora. In:Finegold SM (ed) Clostridia in gastrointestinal disease. Orlando: CRC Press, 1988:1-35.

Goldner SB, Solberg M, Jones S, Post LS (1986) Enterotoxin synthesis by nonsporulating cultures of *Clostridium perfringens*. Appl Env Microbiol 52:407-412.

Gorbach SL, Tabaqchali S (1969) Bacteria, bile and the small bowel. Gut 10:963-972.

Guyton AC (1987) Human physiology and mechanisms of disease. 4th ed. Philadelphia: Saunders WB pp 497-509.

Hammons JL, Jordan WE, Stewart RL, Taulbee JD, Berg RW (1988) Age and diet effects on fecal bile acids in infants. J Pediatr Gastroenterol 7:30-38.

Hatheway CL (1990) Toxigenic Clostridia. Clin Microbiol Rev 3:66-98.

Henderson B, Poole S, Wilson M (1996) Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol Rev 60:316-341.

Heredia NL, Labbe RG, Rodriguez MA, Garcia-Alvarado JS (1991) Growth, sporulation and enterotoxin production by *Clostridium perfringens* type A in the presence of human bile salts. FEMS Microbiol Lett 84:15-22.

Hickey CS, Johnson MG (1981) Effects of pH shifts, bile salts, and glucose on sporulation of *Clostridium perfringens* NCTC 8798. Appl Environ Microbiol 41:124-129.

Hirano T (1992) Interleukin-6 and its relation to inflammation and disease. Clin Immunol Immunopath 62:S60-S65.

Holdsworth RJ (1992) Fatal postoperative gastric necrosis caused by *Clostridium perfringens*. Eur J Surgery 158:447-449.

Holt JG (1984) Bergey's Manual of Systemic Bacteriology. 4th ed. Williams and Wilkins, Baltimore, Md.

Hulkower KI, Wnek AP, McClane BA (1989) Evidence that alterations in small molecule permeability are involved in the *Clostridium perfringens* type A enterotoxin-induced inhibition of macromolecular synthesis in Vero cells. J Cell Physiol 140:498-504.

Johnson S, Gerding DN (1997) Enterotoxemic infections. In Rood JI, McClane BA, Songer JG, Titball RW (eds) The Clostridia: molecular biology and pathogenesis. San Diego: Academic press, Inc. pp. 120-135.

Katahira J, Inoue N, Horiguchi Y, Matsuda M, Sugimoto N (1997) Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin. J Cell Biol 136:1239-1247.

Keller AM (1997) Distribution and detection of *Clostridium perfringens* type A enterotoxin after intraperitoneal and intragastric administration using the murine model. Doctoral Thesis, University of Florida.

Klein E (1895) Uber einen pathgenen anaeroben Darmbacillus, *Bacillus enteritidis sporogenes*. Zentrabl Bakteriol Parasitenkd Infectionskr Hyg 18:737.

Kokai-Kun JF, McClane BA (1997a) The *Clostridium perfringens* enterotoxin. In: Rood JI, McClane BA, Songer JG, Titball RW (eds) The Clostridia: molecular biology and pathogenesis. San Diego: Academic press, Inc. pp. 325-357.

Kokai-Kun JF, McClane BA (1997b) Deletion analysis of the *Clostridium perfringens* enterotoxin. Infect Immun 65:1014-1022.

Krakauer, Fleischer B, Stevens DL, McClane BA, Stiles BG (1997) *Clostridium perfringens* enterotoxin lacks superantigenic activity but induces an IL-6 response form human peripheral blood mononuclear cells. Infect Immun 65:3485-3488.

Krous HF (1984) The microscopic distribution of intrathoracic petechiae in sudden infant death syndrome. Arch Path Lab Med 108:77-79.

Labbe RG, Duncan CL (1977) Evidence for stable messenger ribonucleic acid during sporulation and enterotoxin synthesis by *Clostridium perfringens* type A. J Bacteriol 129:843-849.

Labbe RG, Nolan LL (1981) Stimulation of *Clostridium perfringens* enterotoxin formation by caffeine and theobromine. Infect Immun 34:50-54.

Labbe RG (1989) *Clostridium perfringens.* In: Doyle MP (ed) Food-borne bacterial pathogens. New York: Marcel Dekker, Inc., pp 192-234.

Larson HE, Borriello SP (1988) Infectious diarrhea due to Clostridium perfringens. J Infect Dis 157:390-391.

Lehmann V, Freudenberg MA, Galanos C (1987) Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and d-galactosamine-treated mice. J Exp Med 165:657-663.

Lindsay JA (1996) *Clostridium perfringens* Type A enterotoxin (CPE): more than just explosive diarrhea. Crit Rev Microbiol 22:257-277.

Lindsay JA, Dennison JD (1986) Histopathological effect of *Clostridium perfringens* 8-6 enterotoxin on rabbit intestine. Curr Microbiol13:61-66.

Lindsay JA, Johnson HM, Wallace FM, Soos JM (1994) Can superantigens trigger sudden infant death syndrome? Med Hypoth 43:81-85.

Lindsay JA, Mach AS, Wilkinson MA, Martin LM, Wallace FM, Keller AM, Wojciechowski LM (1993) *Clostridium perfringens* Type A cytotoxic-enterotoxin(s) as triggers for death in the sudden infant death syndrome: development of a toxico-infection hypothesis. Curr Microbiol 27:51-59.

Locket PL, Gallager DD (1989) An improved procedure for bile acid extraction and purification. Lipids 24:221-223.

Lowry OH, Rosenbrough NJ, Randall RJ (1951) Protein measurement with the folin reagent. J Biol Chem 193:265-275.

Mach AS, Lindsay JA (1994) Activation of *Clostridium perfringens* cytotoxic enterotoxin(s) *in vivo* and *in vitro*: role in triggers for sudden infant death. Curr Microbiol 28:261-267.

Mahony DE, Ahmed R, Jackson SG (1992) Multiple typing techniques applied to a Clostridium perfringens food poisoning outbreak. J Appl Bacteriol 72:309-314.

Mannel D, Murray C, Risau W, Clauss M (1996) Tumor necrosis: factors and principles. Immunol Today 17:254-256.

Mantovani A, Bussolino F, Inrona M (1997) Cytokine reulation of endothelial cell function: from the molecular level to the bedside. Trends Immunol 18:231-240.

Marrack P, Blackman M, Kushnir E, Kappler J (1990) The toxicity of staphylococcal enterotoxin B is mediated by T cells. J Exp Med 171:455-464.

Marshall JS, Bienenstock J, Perdue MH, Stanisz AM, Stead RH, Ernst PB (1989) Novel cellular interactions and networks involving the intestinal immune system and its microenvironment. APMIS 97:383-394.

McClane BA (1994) *Clostridium perfringens* enterotoxin acts by producing small molecule permeability alterations in plasma membranes. Toxicol 87:43-67.

McClane BA, Hanna PC, Wnek AP (1988) *Clostridium perfringens* enterotoxin. Microbial Pathogenesis 4:317-323.

McClane BA, McDonel JL (1979) The effects of *Clostridium perfringens* enterotoxin on morphology, viability, and macromolecular synthesis in Vero cells. J Cell Physiol 99:191-200.

McClane BA, Wnek AP, Whitaker-Dowling P (1987) Interferon pretreatment enhances the sensitivity of Vero cells to *Clostridium perfringens* type A enterotoxin. Microbial Pathogenesis 3:195-206.

McClane BA (1996) An overview of *Clostridium perfringens* enterotoxin. Toxicon 34:1343-1355.

McClung LS (1945) Human food poisoning due to growth of *Clostridium perfringens* (*C. welchii*) in freshly cooked chickens. J Bacteriol 50:229-231.

McDonel JL (1980a) Binding of *Clostridium perfringens* [^{125}I] enterotoxin to rabbit intestinal cells. Biochemistry 19:4801-4808.

McDonel JL (1980b) Mechanism of action of *Clostridium perfringens* enterotoxin. Food Tech 12:91-95.

McDonel JL, Asano T (1975) Analysis of unidirectional fluxes of sodium during diarrhea induced by *Clostridium perfringens* enterotoxin in the rat terminal ileum. Infec Immun 11:526-529.

McDonel JL, Duncan CL (1975) Histopathological effects of *Clostridium perfringens* enterotoxin in the rabbit ileum. Infect Immun12:1214-1218.

McDonel JL, McClane BA (1979) Binding versus biological activity of *Clostridium perfringens* enterotoxin in vero cells. Biochem Biophys Res Commun 87:497-504.

Meer RR, Songer JG, Park DL (1997) Human disease associated with *Clostridium perfringens* enterotoxin. Rev Environ Contam Toxicol 150:75-94.

Melville SB, Collie RE, McClane BA (1997) Regulation of enterotoxin production in *Clostridium perfringens*. In Rood JI, McClane BA, Songer JG, Titball RW (eds) The Clostridia: molecular biology and pathogenesis. San Diego: Academic Press, Inc. pp. 471-487.

Miethke T, Wahl C, Heeg K, Echtenacher B, Krammer PH, Wagner H (1992) T cell-mediated lethal shoke in mice by superantigen staphylococcal enterotoxin B:critical role of tumor necrosis factor. J Exp Med 175:91-98.

Miethke T, Wahl C, Regele D, Gaus H, Heeg K, Wagner H (1993) Superantigen mediated shock: a cytokine release syndrome. Immunobiol 189:270-284.

Mietzner RA, Kokai-Kun JF, Hanna PC, McClane BA (1992) A conjugated synthetic peptide corresponding to the C-terminal region of *Clostridium perfringens* type A enterotoxin elicits an enterotoxin neutralizing antibody response in mice. Infect Immun 60:3947-3951.

Mire-Sluis A, Thorpe R (1998) Cytokines. Academic Press, San Diego, Ca.

Misfeldt ML, Legaard PK, Howell SE, Fornella MH, LeGrand RD (1990) Induction of interleukin-1 from peritoneal macrophages by *Pseudomonas aeruginosa* exotoxin A. Infect Immun 58:978-982.

Miwatani T, Honda T, Arai S, Nakayama A (1978) Mode of lethal activity of *Clostridium perfringens* Type A enterotoxin. Japan J Med Sci Biol 31:221-223.

Mondino A, Khoruts A, Jenkins MK (1996) The anatomy of T-cell activation and tolerance. Proc Natl Acad Sci (USA) 93:2245-2252.

Morikawa A, Sugiyama T, Kato Y, Koide N, Jiang G-Z, Takahashi K, Tamada Y, Yokochi T (1996) Apoptotic cell death in the response of D-galactosamine-sensitized mice to lipopolysaccharide as an experimental endotoxic shock model. Infect Immun 64:734-738.

Morin MD, Hopkins WJ (1998) Treatment of mice with staphylococcal enterotoxin B enhances resolution of an induced *Escherichia coli* urinary tract infection and stimulates production of proinflammatory cytokines. Infect Immun 66:2466-2470.

Munoz C, Misset B, Fitting K, Bleriot J-P, Cavaillon J-C, Cavaillon J-M (1991) Dissociation between plasma and monocyte-associated cytokines during sepsis. Eur J Immunol 21:2177-2184.

Murrell WG (1989) *Clostridium perfringens*. In: Buckle K (ed) Food-borne microorganisms of public health significance. Sydney, Australia: AIFST, pp 209-232.

Nagata K, Okamura H, Kunitoh, Uemura T (1997) Mitogenic activity of *Clostridium perfringens* enterotoxin in human periferal lymphocytes. J Vet Med Sci 59:5-7.

Nakano M, Onzuka K, Yamasu H, Zhong WF, Nakano Y (1992) Protective effects of cytokines in murine salmonelosis. Microbial Infect 18:89-95.

Nathan CF, Murray HW, Wiebe ME, Rubin BY (1983) Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J Exp Med 158:670-689.

Newbould MJ, Malam J, McIllmurray JM, Morris JA, Telford DR, Barson AJ (1989) Immunohistological localization of staphylococcal toxic shock syndrome toxin (TSST-1) antigen in sudden infant death syndrome. J Clin Pathol 42:935-939.

Nguyen DT, Eskandari MK, DeForge LE, Raiford CL, Strieter RM, Kunkel SL, Remick DG (1990) Cyclosporin A modulation of tumor necrosis factor gene expression and effects in vitro and in vivo. J Immunol 144:3822-3828.

Ohishi I, Yamamoto K, Sakaguchi G (1981) Evidence for intestinal absorption of *Clostridium perfringens* enterotoxin. FEMS microbiol Lett 12:369-372.

Okewole PA, Itodo AE, Oyetunde IL, Chima JC, Irokanulo EA, Ocholi RA (1991) *Clostridium perfringens* Type A enterotoxemia in pigs: a report of five cases. Brit Vet J 147:484-485.

Onderdonk AB, Allen SD (1995) *Clostridium*. In Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (eds) Manual of Clinical Microbiology (6th ed). Washington, D.C. ASM Press pp. 574-586.

Owens T, Renno T, Taupin V, Krakowski M (1994) Inflammatory cytokines in the brain: does the CNS shape immune responses? Immunol Today 15:566-571.

Ozutsumi K, Sugimoto N, Matsuda M (1987) *Clostridium perfringens* Type A enterotoxin induces release of noradrenaline from the neurosecratory PC12 cell line. BBRC 144:217-223.

Palay DA, Cluff CW, Wentworth PA, Zeigler HK (1986) Cyclosporin inhibits macrophage-mediated antigen presentation. J Immun 136:4348-4353.

Phillips KD (1986) A sporulation medium for *Clostridium perfringens*. Lett Appl Microbiol 3:77-79.

Pousset F (1994) Cytokines as mediators in the central nervous system. Biomed Pharmacother 48:425-431.

Prud'homme GJ, Vanier LE (1993) Cyclosporin, tolerance, and autoimmunity. Clin Immunol Immunopath 66:185-192.

Pullen AM, Potts W, Wakeland EK, Kappler J, Marrack P (1990) Surprisingly uneven distribution of the T cell receptor V beta repertoire in wild mice. J Exp Med 171: 49-62.

Roda A, Cerre C, Simoni P, Ploimeni C, Vaccari C, Pistillo A (1992) Determination of free and amidated bile acids by high-performance liquid chromatography with evaporative light scattering mass detection. J Lipid Res 33:1393-1402.

Rood JI, Cole ST (1991) Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol Rev 55:621-648.

Saito M (1990) Production of enterotoxin by *Clostridium perfringens* derived from humans, animals, foods, and the natural environment in Japan. J Food Prot 53:115-118.

Samuel SC, Hancock P, Leigh DA (1991) An investigation into *Clostridium perfringens* enterotoxin-associated diarrhoea. J Hospital Infect 18:219-230.

Scott CL, Iyasu S, Rowley D, Atrash HK (1998) Postneonatal mortality surveillance--United States, 1980-1994. MMWR 47(SS-2): 15-30.

See RH, Kum WWS, Chang AH, Goh SH, Chow AW (1992) Induction of tumor necrosis factor and interleukin-1 by purified staphylococcal toxic shock syndrome toxin 1 requires the presence of both monocytes and T lymphocytes. Infec Immun 60:2612-2618.

Sen GC (1997) The interferons. In: Remick DG, Frieland JS (eds) Cytokines in health and disease. New York.: Marcel Dekker, Inc pp 199-208.

Setchell KDR, Lawson AM, Tanida N, Sjovall J (1983) General methods for the analysis of metabolic profiles of bile acids and related compounds in feces. J Lipid Res 24:1085-1100.

Shireman S, Klein E, McClane BA (1994) *Clostridium perfringens* Type A enterotoxin induces tissue damage and fluid accumulation in rabbit ileum. J Diarrhoeal Dis Res 12:200-207.

Sivan Y, She G, Schonfeld T, Nitzan M, Nutman J (1992) Sudden infant death in the Tel Aviv and Petah districts. Israel J Med Sci 28:430-435.

Skjelvale R, Tolleshaug H, Jarmund T (1980) Binding of enterotoxin from *Clostridium perfringens* type A to liver cells *in vivo* and *in vitro*. Acta Path Microbiol Scand Sect B 88:95-102.

Skjelkvale R, Uemura T (1977) Experimental diarrhea in human volunteers following oral administration of *Clostridium perfringens* enterotoxin. J Appl Bacteriol 46:281-286.

Slazyk WE, Speirto FW (1990) Liquid-chromatographic measurement of biopterin and neopterin in serum and urine. Clin Chem 36:1364-1368.

Songer JG (1996) Clostridial enteric diseases of domestic animals. Clin Microbiol Rev 9:216-234.

Spooner CE, Markowitz NP, Saravolatz LD (1992) The role of tumor necrosis factor in sepsis. Clin Immunol Immunopath 62:S11-S17.

Stark PL, Lee A (1982) Clostridia isolated form feces of infants during the first year of life. J Pediatr 100:362-365.

Sugii S, Horiguchi Y (1988) Identification and isolation of the binding substance for *Clostridium perfringens* enterotoxin on Vero cells. FEMS Microbiol Lett 52:85-90.

Tadeda K, Matsumoto T, Miyazaki S, Yamaguchi K (1996) Lipopolysaccharide-induced lethality and cytokine production in aged mice. Infect Immun 64:769-774.

Telford DR, Morris JA, Hughes P, Conway AR, Lee S, Barson AJ (1989) The nasopharyngeal flora in SIDS. J Infect 18:125-130.

Thomson A (1998) The Cytokine Handbook (Third edition). Academic Press, San Diego, Ca.

Timmerman CP, Mattsson E, Martinez-Martin L, deGraaf L, van Strijp JAG, Verbrugh HA, Fleer A (1993) Induction of release of tumor necrosis factor from human monocytes by staphlococci and staphylococcal peptidoglycans. Infec Immun 61:4167-4172.

Tocci MJ, Schmidt JA (1997) Interleukin-1: structure and function. In: Remick DG, Frieland JS (eds) Cytokines in health and disease. New York.: Marcel Dekker, Inc pp 1-27.

Todd ECD (1989) Preliminary estimates of costs of food-borne disease in the United States. J Food Prot 52:595-601.

Torres BA, Johnson HM (1994) Identification of an HIV-1 NEF peptide that binds to HLA class II antigens. Biochem Biophys Res Commun 200:1059-1065.

Tracey KJ (1997) Tumor necrosis factor. In: Remick DG, Frieland JS (eds) Cytokines in health and disease. New York.: Marcel Dekker, Inc pp 223-239.

Tracey KJ, Cerami A (1993) Tumor necrosis factor, other cytokines and disease. Annu Rev Cell Biol 9:317-343.

Tsai C-C, Reimann HP (1975) Food poisoning signs in mice induced orally by *Clostridium perfringens* Type A enterotoxin. J Formosan Med Assoc 74:310-315.

Ushijima T (1986) Glycocholic and taurocholic acids as potent sporulation stimulating agents of *Clostridium perfringens*. Medicin Biol (Japan) 113:193-195.

Vege A, Rognum T, Scott H, Aasen A, Saugstad O (1995) SIDS cases have increased levels of interleukin-6 in cerebrospinal fluid. Acta Paediatr 84:193-196.

Vlahcevic ZR, Bell CC, Buhac I, Farrar JT, Swell L (1970) Diminished bile acid pool size in patients with gallstones. Gastroenterol 59:165-173.

Walker PD, Short JA, Roper G (1975) Location of antigens on ultrathin sections of sporeforming bacteria. In: Gerhardt RN, Costilow RN, Sadoff HL (eds) Spores IV American Society for Microbiology pp 572-579.

Wallace FM, Mach AS, Keller AM, Lindsay JA (1999) Evidence for *Clostridium perfringens* enterotoxin (CPE) inducing a mitogenic and cytokine response in vitro and a cytokine reponse in vivo. Curr Microbiol (in press).

Watkins JB, Szczepanik P, Gould JB, Klein A, Lester R (1975) Bile metabolism in the human premature infant. Gastroenterol 67:706-713.

Weiss KF, Strong DH, Groom RA (1966) Mice and monkeys as assay animals for *Clostridium perfringens* food poisoning. Appl Microbiol 14:479-485.

Wewers MD (1997) Cytokines and macrophages. In: Remick DG, Frieland JS (eds) Cytokines in health and disease. New York: Marcel Dekker, Inc pp 339-355.

Wieckowski EU, Wnek AP, McClane BA (1994) Evidence that an ~50 kDa mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specifically bound *Clostridium perfringens* enterotoxin. J Biol Chem 269:10838-10848.

Wilkinson MA (1991) The sudden infant death syndrome in Florida: an epidemiological, pathological, and microbiological study. Masters Thesis, University of Florida.

Williams RC, Showalter R, Kern F (1975) *In vivo* effects of bile salts and cholestyramine on intestinal anaerobic bacteria. Gastroenterol 69:483-491.

Willinger M (1989) SIDS: a challenge. J NIH Res 1:73-80.

Wilson KH, Kennedy MJ, Fekety FR (1982) Use of sodium taurocholate to enhance spore recovery on a medium selective for *Clostridium difficile*. J Clin Microbiol 15:443-446.

Wnek AP, McClane BA (1986) Comparison of receptors for *Clostridium perfringens* type A and cholera enterotoxins in isolated rabbit intestinal brush border membranes. Microbial Patho 1:89-100.

Yachie A, Nobuhiko T, Ohta K, Uehara T, Fujita S-I, Miyawaki T, Taniguchi N (1992) Defective production of interleukin 6 in very small premature infants in response to bacterial pathogens. Infect Immun 60:749-753.

Zar JH (1984) Biostatistical Analysis. Prentice-Hall, New York, NY.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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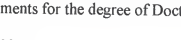

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